University of Alberta

Identification of a Role for the *Saccharomyces cerevisiae* β-karyopherin Kap123p in Influencing Microtubule Stability

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

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For Grandma A.

Abstract

Karyopherins mediate the exchange of macromolecules between the nucleus and the cytoplasm. We propose a link between Kap123p and microtubule function. Null mutant *kap123Δ* strains display sensitivity to microtubule-destabilizing drugs such as benomyl or nocodazole. Several pieces of evidence presented here suggest that *kap123Δ* cells are competent to elicit a spindle assembly checkpoint (SAC) arrest in response to nocodazole-induced microtubule disruption. Consistent with a role for Kap123p in microtubule stability, the viability of *kap123Δ* cells is decreased in the absence of the SAC protein Mad2p. This suggests the SAC monitors microtubule defects in *kap123Δ* cells. Additionally, *kap123Δ* cells exhibit increased benomyl sensitivity in the absence of Mad2p. Physical and genetic interactions, including interactions with the α-tubulins Tub1p and Tub3p, are further suggestive of a role for Kap123p in a microtubule function. As well, a population of *kap123Δ* cells has unusual mitotic spindle structures, producing bi- or multi-nucleated cells.

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List of Symbols, Abbreviations and Nomenclature

3D	3-dimensional
5-FOA	5'-fluoroorotic acid
APC	anaphase promoting complex
bp	base pairs
C-terminal	carboxy terminal
CFP	cyan fluorescent protein
cNLS	classical nuclear localization signal
cs	cold sensitive
d	days
D	Dalton
DAPI	4',6'-diamidino-2-phenylindole dihydrochloride
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FRET	fluorescence resonance energy transfer
g	gravitational force
GAP	guanosine triphosphatase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GST	glutathione-S-transferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
h	hour
HA	haemmaglutinin
HRP	horseradish peroxidase
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactoside
k	kilo (1 x 10^3)
Kap(s)	karyopherin(s)
M	moles per litre or Mega (1 x 10^6)
MCC	mitotic checkpoint complex
MAP(s)	microtubule associated protein(s)
MEN	mitotic exit network
mRNA	messenger RNA
MTOC	microtubule organizing centre
N-terminal	amino terminal
NE	nuclear envelope
NES(s)	nuclear export signal(s)
NLS(s)	nuclear localization signal(s)
NPC(s)	nuclear pore complex(es)
Nup(s)	nucleoporin(s)
OD	optical density

ORF	open reading frame
pA	protein A
PA	protein A (coding region)
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	$-\log[H^+]$
RFP	red fluorescent protein
RNA	ribonucleic acid
SAC	spindle assembly checkpoint
SDS	sodium dodecyl sulphate
SGA	synthetic genetic array
SPB(s)	spindle pole body(ies)
SV40	simian virus 40
tRNA	transfer RNA
ts	temperature sensitive
wt	wild type
YFP	yellow fluorescent protein

CHAPTER ONE: INTRODUCTION

The nuclear envelope (NE) of eukaryotic cells forms a selectively permeable barrier isolating the nuclear contents from other cellular environments. It consists of an outer nuclear membrane, which faces the cytoplasm and is continuous with the endoplasmic reticulum (ER), and an inner nuclear membrane which faces the nucleoplasm (see Gant and Wilson, 1997 for review). Within the nucleus are chromosomal DNA, replication and transcription machinery, and structural and regulatory molecules assisting in these and other cellular processes. During mitotic cell division, DNA is replicated to produce sister chromatids which are uniformly segregated between the resultant mother and daughter cells. Accurate chromosome segregation is crucial to prevent the inheritance of incomplete genetic information. Mutations in this process can result in genetic instability, cancers, or other diseases (Hartwell and Weinert, 1989; Murray, 1992). Therefore, chromosome segregation and cytokinesis are highly regulated events within the cell. They are orchestrated by interdependent biochemical signals, coordinating interplay between chromosomes, components of the cytoskeleton, and cell cycle checkpoints (reviewed in Murray, 1992). These, like other cellular systems, require accurate targeting of macromolecules. For example, shuttling of key structural and regulatory proteins into and out of the nucleus must occur with appropriate timing. Even slight alterations in systems regulating cell division can disrupt the delicate cellular balance.

1.1 Nucleocytoplasmic Transport

The exclusive sites of exchange between the nucleus and the cytoplasm are nuclear pore complexes (NPCs). Situated within the pore membrane domain of the NE, where the outer and inner nuclear membranes fuse to form aqueous channels, NPCs facilitate the movement of cargoes into and out of the nucleus. Water, ions, metabolites and small polypeptides can traverse the NPC channel by diffusion. A highly selective, regulated transport system mediates the molecular exchange of larger molecules (> 40 kD) between the nucleus and the cytoplasm (for review see Gorlich and Kutay, 1999; Rout et al., 2003; Suntharalingam and Wente, 2003).

NPCs are capable of facilitating the nuclear exchange of a great deal of macromolecular material. Based on kinetic analysis of model substrates in permeabilized HeLa cells, Ribbeck and Gorlich (2001) estimate that a single NPC is capable of approximately 1000 translocation events per second, facilitating the transport of 100 MD of material in the same time frame. They further estimate that the *in vivo* transport capacity of HeLa cells is in the range of 10-20 MD per NPC per second (Ribbeck and Gorlich, 2001). This transport through the NPC is the outcome of a regulated interplay between stationary and mobile phases of transport (see Gorlich, 1997; Gorlich and Kutay, 1999; Rout and Aitchison, 2001; Weis, 2003). Proteins of the NPC form the stationary phase of transport. The mobile phase is composed of soluble transport factors (Adam et al., 1990; Adam and Gerace, 1991) known as karyopherins (kaps) and their substrates or "cargoes". Kaps interact with cargo bearing appropriate localization signals and transport this cargo through the NPC in response to directional cues provided by the GTPase Ran (Adam et al., 1989; Adam et al., 1990; Melchior et al., 1993; Moore and Blobel, 1993).

1.1.1 The Ran Gradient

Ran (Gsp1p in yeast) is a Ras-like GTPase whose distribution and GTP-binding state varies between the nucleus and the cytoplasm (Bischoff and Ponstingl, 1991b). Ran is predominantly nuclear (Bischoff and Ponstingl, 1991b; Belhumeur et al., 1993), though a small amount is also found in the cytoplasm (Bischoff and Ponstingl, 1991b) where it cycles during nuclear transport (see section 1.1.3). Based on the differential localization of the Ran-guanosine triphosphatase activating protein (GAP) to the cytoplasm and the Ran-guanine nucleotide exchange factor (GEF) to the nucleoplasm (Ohtsubo et al., 1989; Aebi et al., 1990; Bischoff and Ponstingl, 1991a), it has been long hypothesized that a gradient of Ran exists, with Ran-GDP and Ran-GTP found predominantly in the cytoplasm and nucleoplasm, respectively. Recently, Kalab et al. (2002) used fluorescence resonance energy transfer (FRET) to visualize the Ran gradient in *Xenopus* egg extracts. The authors constructed fluorescent probes with an N-terminal yellow fluorescent protein (YFP) domain, a central Ran-GTP binding domain, and a C-terminal cyan fluorescent protein (CFP) domain. In the absence of Ran-GTP, the conformational flexibility of the probe allows CFP to be proximal to YFP so that excitation of CFP produces a "donor" emission which can excite the YFP "acceptor". This results in emission by both CFP and YFP, producing a green fluorescent signal. Conversely, in the presence of Ran-GTP, the flexibility of the probe is decreased and excited CFP cannot provide donor emission for YFP excitation. This results in a blue fluorescent signal. The addition of this probe to *Xenopus* egg extracts released from their cytostatic factor induced arrest and stimulated to enter interphase with $CaCl_2$, followed by CFP excitation, leads to the emission of blue fluorescence from the nucleus and green fluorescence from the cytoplasm (Kalab et al., 2002). This indicates a high Ran-GTP concentration in the nucleus and a low Ran-GTP concentration in the cytoplasm (Kalab et al., 2002). This "Ran gradient" appears to contribute to the organization and directionality of transport through the NPC (Melchior et al., 1993; Moore and Blobel, 1993). GTP-bound Ran in the nucleus interacts with nuclear transport factors and influences the interactions of these transport factors with their substrates and with the NPC (see section 1.1.3).

1.1.2 Nuclear Pore Complexes - The Stationary Phase of Nuclear Transport

Each NPC is a network of individual proteins termed nucleoporins (nups). A characteristic, conserved feature of NPCs is their elegant eight-fold symmetry. Using cryo-electron microscopy, Akey and Radermacher (1993) were able to visualize *Xenopus* NPCs and construct 3D images of NPC architecture. Structurally, the NPC delineates a central channel in a plane perpendicular with the nuclear envelope (NE). Around this central channel or central transporter, nups are arranged in a spoke-ring conformation with four rings in the plane of the NE and eight spokes running parallel to the transport channel (Akey and Radermacher, 1993) (see *Figure 1-1*). The NPC also displays a two-fold pseudo-symmetry in the plane of the NE. Cytoplasmic filaments extend from the cytoplasmic face of the NPC while, on the nuclear face, extensions converge into a nuclear basket structure (Akey and Radermacher, 1993) (*Figure 1-1*).

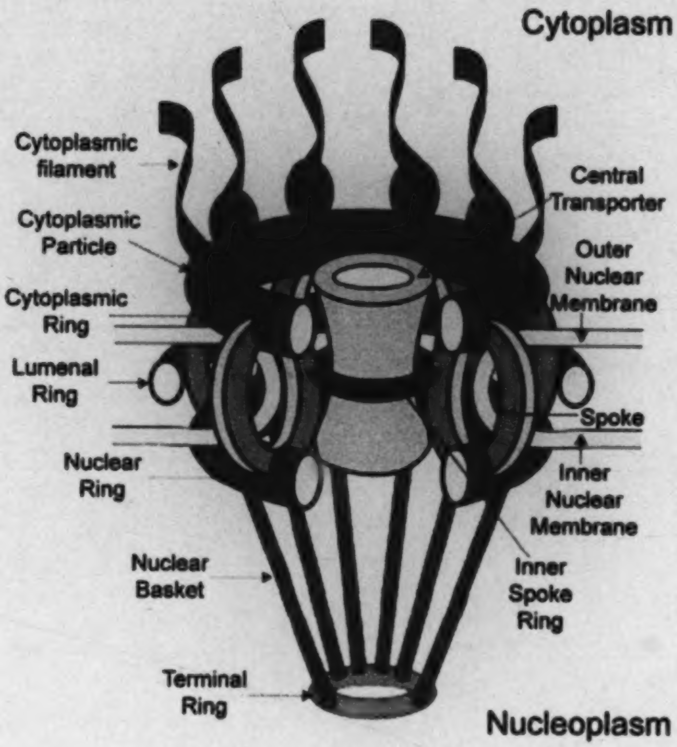

Cytoplasm
Cytoplasmic filament
Central Transporter
Cytoplasmic Particle
Outer Nuclear Membrane
Cytoplasmic Ring
Lumenal Ring
Spoke
Nuclear Ring
Inner Nuclear Membrane
Nuclear Basket
Inner Spoke Ring
Terminal Ring
Nucleoplasm

Figure 1-1. The nuclear pore complex. Model of conserved NPC architecture, based on cryo-electron microscopy of *Xenopus* NPCs (Akey and Radermacher, 1993). A cut-away view exposing internal structures of the NPC is shown. Illustrated are the spoke-ring conformations of the NPC, as well as the 8-fold symmetry of the NPC. 8 spokes are assembled around the central transporter in a plane perpendicular to the NE. Cytoplasmic filaments and a nuclear basket extend into the cytoplasm and nucleoplasm, respectively. Modified from Suntharalingam and Wente (2003).

Though complex and massive structures (~44 MD in yeast (Rout et al., 2000) and ~60 MD in vertebrate (Cronshaw et al., 2002) cells), NPCs are composed of relatively few unique proteins. In fact, proteomic analysis of NPCs suggests only about 30 distinct nups make up the intricate structure of yeast (Rout et al., 2000) and vertebrate (Cronshaw et al., 2002) NPCs. Stoichiometric analysis suggests each nup is present in one, two to four copies per spoke, forming repetitive sub-structures (Rout et al., 2000; Rout and Aitchison, 2001). Most nups have a symmetrical distribution, although the structural differences between the nuclear and cytoplasmic faces of the NPC reflect the biased distribution of a few nups to either the nuclear or cytoplasmic sides of the NPC (Rout et al., 2000). Nups have been categorized into three groups: FG-repeat nups, non-FG repeat nups and poms (reviewed in Rout and Aitchison, 2001; Suntharalingam and Wente, 2003). FG-nups contain repeats of the phenylalanine-glycine motif(s) FXFG, GLFG, PSFG or FG. These nups interact with kaps and are integral to nuclear transport models (Ribbeck and Gorlich, 2001; Rout and Aitchison, 2001). Non-FG repeat nups apparently serve a more structural role for the NPC. It is postulated that these nups form the NPC framework, participating in NPC assembly (Siniossoglou et al., 1996; Zabel et al., 1996; Boehmer et al., 2003; Harel et al., 2003; Walther et al., 2003a). Poms ("Pore Membrane" nucleoporins) are integral membrane proteins believed to anchor the NPC in the NE (Doye and Hurt, 1997; Rout and Aitchison, 2001).

1.1.3 The Mobile Phase of Nuclear Transport

In vitro transport assays using digitonin-permeabilized HeLa cells aided in the identification of cytosolic factors required for nuclear transport (Adam et al., 1990).

These soluble transport factors, called karyopherins (kaps; also known as importins, exportins, or transportins), have subsequently been characterized on the basis of their interactions with transport substrates (cargo), nups and Ran. Kaps transport cargo into or out of the nucleus based on the recognition of specific nuclear localization signals (NLSs) or nuclear export signals (NESs) found on their cargo (Adam et al., 1989; Adam and Gerace, 1991). The "classical" nuclear localization signal (cNLS) consists of one to two clusters of basic amino acid residues, as in the nuclear targeting signal identified for the SV40 large T antigen (Dingwall and Laskey, 1991; Gorlich, 1997). Import of cNLS containing proteins is mediated by Kap-α and Kap-β1 (Kap60p and Kap95p in yeast) which form a heterodimer. Kap-α recognizes the cNLS-bearing cargo and Kap-β1 binds FG-nups and Ran (Adam and Gerace, 1991; Moore and Blobel, 1993; Moroianu et al., 1995a). Other β-kaps which interact directly with their cargo have subsequently been identified, based on their similarity to Kap-β1/Kap95p and their ability to bind Ran (Gorlich et al., 1997; Wozniak et al., 1998). The N-terminal regions tend to be similar between β-kap family members and are hypothesized to represent regions responsible for Ran and FG-nup binding. Divergent areas may facilitate specific interactions with distinct transport substrates and nups (Wozniak et al., 1998; Weis, 2003).

At least 14 β-kaps have been identified in *S. cerevisiae* and ~22 in metazoans (reviewed in Wozniak et al., 1998; Strom and Weis, 2001; Weis, 2003). Yeast β-kaps involved in nuclear import are: Kap95p/Kap-β1 (with Kap60p/Kap-α), Kap104p, Kap121p/Pse1p, Kap123p, Kap114p, Sxm1p/Kap108p, Nmd5p, Mtr10p, and Kap122p. Other kaps, such as Cse1p, Los1p, and Crm1p mediate nuclear export, while Msn5p participates in both import and export (Wozniak et al., 1998; Strom and Weis, 2001; Yoshida and Blobel, 2001; Weis, 2003). Each kap appears to mediate the transport of one or more distinct class(es) of cargo (Aitchison et al., 1996; Gorlich, 1997; Rout et al., 1997; Wozniak et al., 1998). Furthermore, these cargo repertoires tend to be conserved between related kaps from different species. Kap104p and its mammalian counterpart Kap-β2 (transportin) transport mRNA associated proteins containing an M9 NLS, a 38 amino acid sequence first identified in the mammalian mRNA regulatory protein A1 (Siomi and Dreyfuss, 1995; Aitchison et al., 1996; Bonifaci et al., 1997; Wozniak et al., 1998; Lee and Aitchison, 1999). Sxm1p imports tRNA maturation factors (Rosenblum et al., 1997), while Kap122p imports transcription factors (Titov and Blobel, 1999). Kap121p also mediates the import of certain transcription factors (Kaffman et al., 1998; Leslie et al., 2002). As well, both yeast and human Kap121p (Kap-β3) import ribosomal and ribosomal biogenesis proteins (Rout et al., 1997; Yaseen and Blobel, 1997; Leslie et al., 2004). Kap123p (Kap-β4) also mediates ribosomal protein import (Rout et al., 1997; Schlenstedt et al., 1997). Yeast Kap123p also imports at least one ribosomal protein export regulator (Sydorskyy et al., 2003) and interacts with histones H3 and H4 (Mosammaparast et al., 2002) and the core protein of hepatitis C virus (Isoyama et al., 2002).

Interestingly, many of the genes coding yeast kaps are non-essential (Wozniak et al., 1998). In the absence of a particular kap, mislocalization of its cargo could lead to severe cellular defects if the cargo repertoire includes one or more proteins whose nuclear import or export is a prerequisite for an essential cellular process. In order for cells to function under conditions of kap mutation or deletion with minimal detrimental effects, additional pathways must exist to accomplish the nuclear import and/or export of indispensable transport substrates. Indeed, numerous overlapping kap-mediated transport pathways have been identified. For example, Kap104p has the ability to transport a subset of Kap121p substrates in the presence of *kap121* mutations (Leslie et al., 2004). In a *kap123Δ* mutant, Kap121p exhibits increased interactions with Kap123p ribosomal transport substrates and appears to substitute for Kap123p in this import pathway (Rout et al., 1997). As well, Kap123p and Kap121p appear to have overlapping nuclear import pathways for the histones H3 and H4 (Mosammaparast et al., 2002). Reflective of these functional overlaps, synthetic fitness defects exist between *kap123Δ* and *kap121* mutants (Sydorskyy et al., 2003). Genetic interactions between *KAP123* and *SXM1/KAP108* and *NMD5/KAP119* (Sydorskyy et al., 2003) suggest further overlap for Kap123p-mediated transport pathways.

Nuclear transport appears to occur through a multi-step process, with transport substrates docking at the NPC periphery before being translocated through the central transporter (Akey and Goldfarb, 1989). Kaps mediate these cargo interactions with the NPC. Importing kaps interact with their cargo in the cytoplasm then associate with nups at the cytoplasmic face of the NPC. Following translocation across the NPC (see section 1.2) the kap/cargo complex encounters nucleoplasmic Ran-GTP. Ran-GTP interacts with β-kaps (Rexach and Blobel, 1995), apparently inducing kap conformational changes (Gorlich et al., 1996; Moroianu et al., 1996; Mosammaparast and Pemberton, 2004) which result in the dissociation of the kap/cargo complex. This leads to delivery of cargo to the nucleoplasm (see *Figure 1-2*). Conversely, exporting kaps bound to Ran-GTP display enhanced interactions with cargo to be exported (Richards et al., 1997). Exporting kaps interact with nups on the nuclear face of the NPC and are translocated though the NPC. Following export, Ran-GTP is hydrolysed to Ran-GDP in the cytoplasm by the Ran-GAP. Again, this changes the conformation of β-kaps and results in cargo release (*Figure 1-2*). Consistent with this model, kaps mediating nuclear export (exportins) appear to exhibit enhanced binding to FG-nups in the presence of Ran-GTP (Rexach and Blobel, 1995; Floer and Blobel, 1999; Seedorf et al., 1999; Allen et al., 2001). This favours kap/cargo interactions with the NPC and movement of exporting kap/cargo complexes out of the nucleus. Conversely, kaps mediating nuclear import (importins) display decreased FG-nup interactions in the presence of elevated Ran-GTP (Seedorf et al., 1999; Allen et al., 2001) which should encourage release of the kap/cargo complex from the NPC into the nucleoplasm.

The importance of Ran in nuclear transport cycles necessitates its maintenance at adequate levels in the nucleoplasm. Since Ran-GTP is translocated out of the nucleus bound to exporting kaps, nuclear levels of Ran would be rapidly depleted were there no mechanism to restore it to the nucleus (*Figure 1-2*). Shuttling of Ran from the cytoplasm to the nucleus is accomplished by the protein nuclear transport factor 2 (Ntf2p)/p10 (Moore and Blobel, 1994; Paschal and Gerace, 1995; Nehrbass and Blobel, 1996; Ribbeck et al., 1998; Smith et al., 1998) (*Figure 1-2*). To date, Ran is the only cargo identified for Ntf2p (reviewed in Weis, 2003).

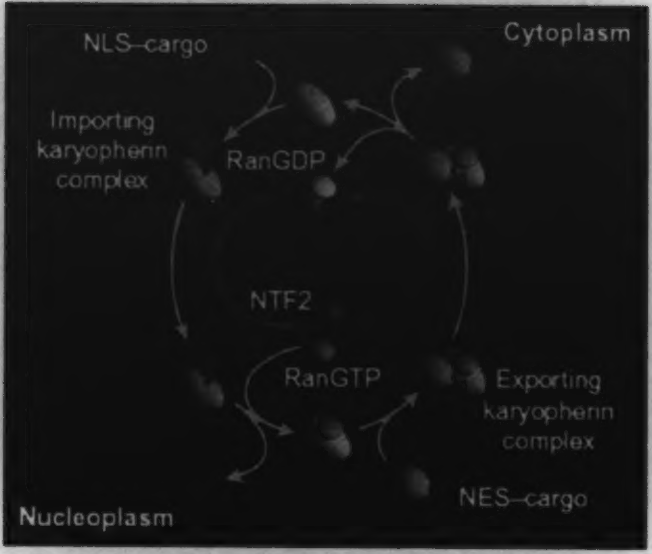

NLS–cargo
Cytoplasm
Importing karyopherin complex
RanGDP
NTF2
RanGTP
Exporting karyopherin complex
NES–cargo
Nucleoplasm

Figure 1-2. Cycle of nuclear transport. An illustration of kap-mediated transport between the cytoplasm and the nucleoplasm. An importing kap interacts with cargo bearing an appropriate nuclear localization signal (NLS) in the cytoplasm and facilitates transport of the kap/cargo complex through the nuclear pore complex (NPC), shown schematically in blue. Ran-GTP binding to the importing β-kap releases cargo in the nucleoplasm. By a reciprocal process, an exporting kap bound to Ran-GTP interacts with cargo bearing an appropriate nuclear export signal (NES) in the nucleoplasm and the exporting kap/cargo complex moves through the NPC into the cytoplasm. The cytoplasmic Ran-GAP facilitates Ran-mediated hydrolysis of GTP to GDP, resulting in cargo release into the cytoplasm. Ran is recycled into the nucleus by NTF2. Modified from Rout et al., 2003.

1.2 Models of Transport

Though key transport machinery has been identified and the basic principles of transport elucidated, little is certain about the exact mechanism by which kap/cargo complexes move through the NPC. A number of models have been proposed and two are discussed here.

1.2.1 The Brownian Affinity Gate Model

The Brownian affinity gate or virtual gate model proposes that the NPC forms an entropic barrier to transport (Rout et al., 2000; Rout et al., 2003). Randomly diffusing molecules in the cytoplasm or nucleoplasm are capable of moving in numerous directions. Hence, the probability that a molecule will move into and through the restrictive channel of the NPC is relatively small. Furthermore, cytoplasmic filaments around the entrance to the NPC, as well as FG-nups lining the central transporter, decrease the space available for molecular diffusion (Rout et al., 2003). In order to navigate through the NPC, molecules must exploit opportunities to interact physically with the NPC structure. Here, the importance of kap-FG-nup interactions is evident (Moroianu et al., 1995b; Rout et al., 2003). Low affinity interactions between kaps and FG-nups encourage the movement of cargo complexes into and through the NPC with a high on/off rate, allowing translocation through the pore to proceed rapidly (Rout et al., 2003).

1.2.2 The Selective Phase Model

The selective phase model also relies on FG-nups for its explanation of transport, though a different mechanism is proposed. The selective phase model argues that hydrophobic interactions occurring between phenylalanine groups of FG-nups form an impermeable barrier or meshwork of nups (Ribbeck and Gorlich, 2001). In order to pass through the NPC, molecules must become incorporated into this meshwork. Theoretically, interactions between translocating kap/cargo complexes and FG-nups can compete with nup-nup interactions of this meshwork such that kaps become solublized within the NPC channel (Ribbeck and Gorlich, 2001). In support of this model, it was demonstrated that a number of kaps are capable of binding a hydrophobic interaction column (Ribbeck and Gorlich, 2002). Furthermore, the profile of kaps bound to this hydrophobic interaction column is similar to that of kaps bound to immobilized Ran-GTP (Ribbeck and Gorlich, 2002). These data are consistent with the ability of kaps to be solublized within a hydrophobic network (Ribbeck and Gorlich, 2002). Moreover, it was shown that NPCs can be "opened" by perturbing hydrophobic interactions between FG-nups (Ribbeck and Gorlich, 2002).

1.3 Regulation of Transport

1.3.1 Nup Mediated Transport Regulation

In addition to low affinity interactions, some kaps exhibit more specific, high affinity interactions with nups, forming distinct pathways through the NPC (Marelli et al., 1998; Makhnevych et al., 2003). The implications of these individual routes through the pore are many. These pathways not only increase the potential volume of transport through the NPC at any given time, due to decreased competitive interference (Rout and Aitchison, 2001; Rout et al., 2003), but can also provide regulatory mechanisms for individual transport pathways (Makhnevych et al., 2003). In other words, nup-nup interactions and nup-kap interactions can be reordered to achieve transport regulation. A striking example of the transport regulation made possible by specific reorganization of nup interactions is the cell cycle regulation of Kap121p-mediated transport in *S. cerevisiae* resulting from molecular rearrangements of the Nup53p-containing NPC sub-complex (Makhnevych et al., 2003). Nup53p associates with Nup170p during interphase of the cell cycle, masking the high affinity Kap121p binding site of Nup53p (Lusk et al., 2002). This allows Kap121p and its cargo to proceed through the NPC. Following phosphorylation of Nup53p during mitosis (Marelli et al., 1998), the Nup53p-containing NPC sub-complex undergoes molecular rearrangements (Makhnevych et al., 2003). Nup53p no longer associates with Nup170p. Rather, it binds the nup Nic96p, exposing the Kap121p binding site on Nup53p. Upon docking at Nup53p, Kap121p movement through the NPC is inhibited and Kap121p cargo is released prematurely. It is yet to be determined whether similar regulatory mechanisms exist for transport mediated by other kaps.

1.3.2 Regulation of Kap-Cargo Interactions

Post-translational cargo modification, including phosphorylation or acetylation, are another mechanism by which the nuclear transport of specific substrates is regulated (Kaffman et al., 1998; Madison et al., 2002). For example, the transcription factor Pho4p is dephosphorylated in response to low phosphate levels, leading to its nuclear import by

Kap121p. Conversely, the phosphorylated form of Pho4p is recognized by Msn5p and exported from the nucleus (Kaffman et al., 1998). Other types of cargo modification can also influence kap-cargo interactions. Any change in cargo conformation which masks or exposes nuclear localization or export signals will affect the ability of kaps to recognize transport substrates. Transport of a substrate is reduced as a result of decreased NLS or NES recognition by its cognate kap. Conversely, conformational changes which expose these NLS or NES domains of cargo increase the likelihood of cargo transport (reviewed in Lusk et al., 2004).

1.4 Interactions between the Nuclear Transport System and Cell Cycle Regulators

Nucleocytoplasmic transport is an essential process in all eukaryotes. In organisms such as *S. cerevisiae*, which undergo a closed mitosis, the importance of nucleocytoplasmic transport is further underscored. In the absence of NE breakdown, any macromolecules which require removal from or access to the nuclear compartment must traverse the NPC. Therefore, accurate cell division is intimately linked to functional and efficient nucleocytoplasmic transport. There are numerous examples of nup or kap mutations which result in cell cycle defects (Xiao et al., 1993; Loeb et al., 1995; Kerscher et al., 2001; Asakawa and Toh-e, 2002). A possible explanation is that these mutations disrupt nucleocytoplasmic transport. Mutation to or loss of a particular kap may result in mislocalization of its cognate cargo(es). If efficient cargo localization is required for accurate cell division, this mislocalization can produce cell cycle defects. Nevertheless, it is also possible that these defects reflect nup or kap involvement in processes beyond traditional nuclear transport.

1.4.1 Participation of Nups, Kaps, and Ran in Non-transport Systems

The NPC and other transport machinery have recognized roles in processes outside of nuclear transport. For example, it is evident that proteins of the NPC do more than simply form a channel for nuclear transport. Consistent with their residence in the NE, nups have been implicated in influencing NE biogenesis (Doye and Hurt, 1997; Marelli et al., 2001) and NE breakdown (Liu et al., 2003). The localization of NPCs within the NE also positions nups with the cell division machinery including spindle pole bodies (SPBs), kinetochores, chromatin, and cell cycle checkpoint proteins. This provides nups and interacting kaps opportunities to influence cell cycle regulatory systems.

1.4.2 Interactions between Nups and Cell Cycle Regulatory Proteins

A number of compelling interactions have been identified between nups and cell cycle regulatory proteins. For example, Mad1p, a component of the spindle assembly checkpoint (SAC), physically interacts with the NPC in both yeast (Iouk et al., 2002) and mammalian cells (Campbell et al., 2001). This checkpoint is important for ensuring proper interactions between kinetochore proteins and the mitotic spindle (see section 1.6.1). Thus, several SAC proteins associate with kinetochores (Rudner and Murray, 1996; Chan et al., 1999; Chan et al., 2000; Gillett et al., 2004, R.J. Scott and R.W. Wozniak, unpublished). Similarly, certain nups also localize at kinetochores. In HeLa cells, all nine members of the Nup107-160 complex localize dynamically at kinetochores following mitotic NE breakdown (Belgareh et al., 2001; Loiodice et al., 2004) while RanBP2/ Nup358 localizes to both kinetochores and the mitotic spindle (Joseph et al., 2002; Joseph et al., 2004). These are not the only instances in which there is coincident localization of nups and cell cycle regulatory proteins. Ndc1p localizes with both the NPC and the spindle pole body (SPB) and appears to contribute to both NPC and SPB function (Chial et al., 1998; Lau et al., 2004). In fission yeast, Ned1, a protein influencing chromosome segregation and nuclear morphology, interacts with the essential nup Nup189, a nup bearing sequence homology to the mammalian nup Nup98/96 (Tange et al., 2002). This suggests a relationship between the NPC and chromosome segregation in this organism. In *S. cerevisiae*, a *nup170Δ* mutant displays chromosome segregation defects, suggesting nups may influence chromosome segregation in this organism as well (Kerscher et al., 2001).

1.4.3 Kap Mutations Resulting in Cell Cycle Defects

Functional relationships also exist between kaps and cell cycle regulatory machinery. Reflective of this, phenotypes similar to those of cell division mutants have been demonstrated as a result of certain kap mutations. For example, a mutation in Kap104p overcomes the mitotic arrest of a *cdc15-2* temperature sensitive (ts) strain (Asakawa and Toh-e, 2002). A *cse1/kap109* cold sensitive (cs) mutant was identified in a screen for mutants defective in chromosome segregation. The authors speculated that Cse1p/Kap109p may influence kinetochore interactions with the mitotic spindle (Xiao et al., 1993). The chromosome segregation defect identified in the *cse1-1* strain may also reflect the role of Cse1p in the nuclear export of Kap60p/Srp1p (Kap-α) (Hood and Silver, 1998; Solsbacher et al., 1998). Overexpression of Kap60p rescues the cold sensitivity and partially rescues the chromosome missegregation defects of *cse1-1* cells

(Xiao et al., 1993). Conversely, *kap60/srp1* mutants are defective for progression through mitosis (Loeb et al., 1995).

1.4.4 Ran-mediated Functions beyond Nuclear Transport

It has been postulated that kaps can also act as effectors for non-transport functions of Ran (reviewed in Mosammaparast and Pemberton, 2004). These include NE assembly (Askjaer et al., 2002), NPC assembly (Ryan et al., 2003; Walther et al., 2003b), and regulation of kinetochore-microtubule attachments (Salina et al., 2003; Joseph et al., 2004). For example, altering the levels of the Ran-GAP or of the Ran-GEF, RCC1, in *Xenopus* mitotic extracts leads to the mislocalization of the kinetochore associated SAC proteins and results in SAC abrogation (Arnaoutov and Dasso, 2003; Salina et al., 2003; Joseph et al., 2004). Kinetochore attachment to the mitotic spindle targets RanGAP and RanBP2/Nup358, an accessory factor for Ran-mediated GTP hydrolysis, to the spindle and kinetochores in HeLa cells (Joseph et al., 2002; Joseph et al., 2004). These interactions are proposed to facilitate the assembly of stable kinetochore-microtubule attachments (Salina et al., 2003; Joseph et al., 2004).

Ran also functions in spindle formation. In *Caenorhabditis elegans*, depletion of Ran, RanBP2, Ran-GAP or RCC1 not only leads to defective nuclear envelope reassembly after mitosis, but compromised mitotic spindle formation (Askjaer et al., 2002). The role of Ran in mitotic spindle assembly has been well studied in vertebrate systems (Dasso, 2001; Dasso, 2002). The addition of Ran-GTP to *Xenopus* egg extracts induces the formation of microtubule asters (Carazo-Salas et al., 1999; Ohba et al., 1999; Wilde and

Zheng, 1999), while Kap-α and Kap-β1 inhibit the aster promoting activity of Ran-GTP (Carazo-Salas et al., 2001; Gruss et al., 2001; Nachury et al., 2001). Though the cytoplasmic-nucleoplasmic Ran gradient is abolished during NE breakdown in higher eukaryotes, elements of this system are maintained during mitosis. The chromatin bound Ran-GEF, RCC1 (Ohtsubo et al., 1989; Bischoff and Ponstingl, 1991a; Bischoff and Ponstingl, 1991b), maintains a high concentration of Ran-GTP in the vicinity of chromosomes. Thus, a model has been proposed whereby Ran-GTP mediated release of spindle polymerization and organization factors from Kap-α and Kap-β1 promotes the formation of the mitotic spindle (Carazo-Salas et al., 2001; Gruss et al., 2001; Nachury et al., 2001). In the presence of Ran-GDP, these proteins remain bound to Kap-α and Kap-β1, inhibiting their activity. Upon encountering Ran-GTP near chromatin, proteins are dissociated from Kap-α and Kap-β1, similar to the release of cargoes from importing kaps upon entering the nucleoplasm. In *Xenopus*, microtubule aster promoting proteins include TPX2 ("target protein for *Xenopus* kinesin-like protein 2") (Gruss et al., 2001) and NuMa ("nuclear-mitotic apparatus protein") (Nachury et al., 2001; Wiese et al., 2001). Elements of this system appear to be conserved in other species as well. For example, the mammalian protein NuSAP ("nucleolar spindle-associated protein") induces the formation of microtubule bundles in the presence of Ran-GTP (Raemaekers et al., 2003). Again, this activity is inhibited by Kap-β1 (Raemaekers et al., 2003). These findings illustrate mechanisms by which kap-Ran interactions can be exploited for non-transport purposes.

Though less well characterized, Ran may also influence spindle assembly in organisms undergoing a closed mitosis. A Ran mutant which is competent for nuclear transport but which develops defects in microtubule organization and cell morphology was identified in fission yeast (Fleig et al., 2000). To date, no clear links between the nuclear transport machinery and mitotic spindle organization have been identified in budding yeast, though there are clues that Ran may influence microtubule assembly in this organism as well. The *ntf2-2* ts mutant (Quimby et al., 2000), in which Ran is depleted from the nucleus (Quimby et al., 2005), undergoes a Mad2p-dependent cell cycle arrest at the non-permissive temperature (Quimby et al., 2000). Because Ntf2p recycles Ran into the nucleus and the Mad2p checkpoint monitors spindle formation, these data suggest nuclear depletion of Ran may result in compromised spindle formation, necessitating a SAC arrest.

1.5 Regulation of Microtubule Assembly in *S. cerevisiae*

1.5.1 Microtubule Structure

Mitotic spindle assembly and function rely on the availability of functional microtubules and on proteins assisting in microtubule organization, stability, and dynamics. Microtubules are central components of the cytoskeletal network functioning in processes such as nuclear migration, cell division, and organellar inheritance. They consist of hollow cylinders composed of a tubulin heterodimer of alternating α- and β- tubulin monomers (reviewed in Nogales, 2001). Though a number of α- and β- isoforms have been identified in higher eukaryotes, *S. cerevisiae* has only two α-tubulin genes and one β-tubulin gene (Nogales, 2001). *TUB1* is an essential gene encoding the primary α-tubulin protein, while *TUB3* is a non-essential gene encoding a second α-tubulin protein. The single β-tubulin is encoded by the essential gene *TUB2*.

1.5.2 Microtubule Folding

Before functional tubulin is produced, it undergoes a series of chaperone-assisted folding steps. Among the systems employed in tubulin folding are a hetero-oligomeric complex known as the cytosolic chaperonin CCT (TriC, TCP1) complex, tubulin co-factors and the GimC-complex (Nogales, 2001). Genes encoding proteins of the GimC-complex ("genes involved in microtubule biogenesis") were identified based on genetic interactions with the gene encoding γ-tubulin (Geissler et al., 1998), a protein with microtubule nucleating activity (Sobel and Snyder, 1995; Marschall et al., 1996; Spang et al., 1996). Further characterization revealed that the five components of the GimC-complex, Gim1/Yke1p, Gim2/Pac10p, Gim3p, Gim4p, and Gimp5p function in both actin and tubulin folding and possibly influence microtubule nucleation (Geissler et al., 1998; Siegers et al., 1999). Though none are essential, knocking out any of these genes results in decreased levels of α-tubulin (Geissler et al., 1998). It appears the GimC-complex functions cooperatively with the CCT complex, targeting substrates to the chamber-like CCT complex for subsequent folding (Siegers et al., 1999). Consistent with the roles of the GimC- and CCT-complexes in facilitating the formation of functional tubulin, several GimC and CCT mutants exhibit phenotypes similar to tubulin mutants, including sensitivity to microtubule-destabilizing drugs such as benomyl (reviewed in Geissler et al., 1998; Dunn et al., 2001) and an increased prevalence of binucleated cells versus wild type (Dunn et al., 2001).

1.5.3 Microtubule Nucleation

Fully functional microtubules form only in the presence of an appropriate template. Formation of this template is referred to as microtubule nucleation (Nogales, 2001) and is achieved by a SPB complex called the γ-tubulin complex. Cytoplasmic γ-tubulin complexes also exist in animal cells in association with centrosomal proteins (Draber and Sulimenko, 2003). In yeast, the γ-tubulin complex is composed of γ-tubulin (Tub4p) (Sobel and Snyder, 1995; Marschall et al., 1996; Spang et al., 1996) as well as the SPB components Spc97p and Spc98p (Geissler et al., 1996; Knop et al., 1997). This complex nucleates cytoplasmic (astral) microtubules from the outer plaque or half bridge of the SPB where it is anchored by Spc72p in a cell cycle dependant manner (Knop and Schiebel, 1998; Gruneberg et al., 2000; Segal and Bloom, 2001). Spc110p anchors the Tub4p-complex to the inner plaque of the SPB (Knop and Schiebel, 1997) where it nucleates nuclear microtubules.

1.5.4 Mitotic Spindle Movement

Using time lapse microscopy of cells expressing GFP-tagged α-tubulin, the microtubule movements directing nuclear migration, spindle positioning, and chromosome segregation in budding yeast have been well characterized (Adames and Cooper, 2000). Microtubule actions observed during mitosis include "capture/shrinkage", "sweeping", and "sliding" (Adames and Cooper, 2000). "Capture/shrinkage" describes the process of microtubule capture at the cell cortex followed by microtubule shortening. This leads to spindle movement. Prior to anaphase, capture/shrinkage events and, to a lesser extent, growth of microtubules against the cortex of the mother cell, move the spindle to the bud neck site (Adames and Cooper, 2000). "Sweeping" refers to the lateral movement of the spindle caused by pivoting around one SPB while contacting the cell cortex. Spindle alignment occurs as a result of sweeping by astral microtubules which emanate from the SPB of the pre-anaphase spindle and contact the bud cortex (Adames and Cooper, 2000). Following anaphase, spindle movement and nuclear migration to the bud are achieved by microtubule sliding and, to a lesser degree, capture/shrinkage events between the SPB associated astral microtubules and the bud tip (Adames and Cooper, 2000). "Sliding" refers to the lateral association along the length of the microtubules with the bud cortex which pulls the spindle through the bud neck with the help of dynein and dynactin (Yeh et al., 1995; Adames and Cooper, 2000).

1.5.5 Microtubule Motor Proteins

Assisting in such processes, as well as in spindle separation and stability, are microtubule motors (examples are kinesin-related motor and *bimC* sub-family motor proteins) (Cottingham and Hoyt, 1997; Geiser et al., 1997; Saunders et al., 1997; Adames and Cooper, 2000), cytoplasmic dynein (Sheeman et al., 2003), and additional proteins bridging interactions between SPBs, astral microtubules and the cell cortex. Though microtubule motor proteins mediate many important processes, yeast cells can grow in the absence of five of the seven identified motor proteins (Cottingham et al., 1999), suggesting functional redundancy between these pathways. Though most motor proteins are not essential for cell division, antagonistic actions of motor proteins can lead to subtle defects in the absence of specific motors (Saunders et al., 1997; Segal and Bloom, 2001).

Microtubules display "dynamic instability", alternating between shrinkage-to-growth phases ("rescue") and growth-to-shrinkage phases ("catastrophe") (Kinoshita et al., 2001; Nogales, 2001). "Treadmilling", another dynamic property of microtubules, refers to the net flow of microtubule subunits from one end of the microtubule to the other without changing the overall microtubule length (reviewed in Nogales, 2001). Appropriate timing of these events allows cell cycle mediated changes to microtubule structure and facilitates mitotic spindle assembly, accurate chromosome capture by the mitotic spindle, and anaphase spindle elongation (Hoyt and Geiser, 1996; Straight et al., 1998; Cottingham et al., 1999; Nogales, 2001). Some of the microtubule motors already described, along with other microtubule associated proteins (MAPs), participate in regulation of microtubule dynamics and stability (Kinoshita et al., 2001). For example, the *Xenopus* MAP XMAP215 stabilizes microtubules assembled *in vitro* while the kinesin XKCM1 destabilizes *in vitro* microtubules (Kinoshita et al., 2001).

1.6 Cell Cycle Checkpoints Regulating Spindle Assembly and Position

Mitotic spindle assembly, chromosome capture by the mitotic spindle, spindle positioning and spindle elongation all contribute to accurate chromosome segregation during cell division. Since the faithful distribution of chromosomes to dividing cells is crucial, it is not surprising that the cell employs checkpoints with respect to spindle assembly and positioning (for review see Lew and Burke, 2003). A cell cycle checkpoint is a feedback mechanism by which cells ensure that one process is complete before subsequent cellular processes proceed (Hartwell and Weinert, 1989).

1.6.1 The Spindle Assembly Checkpoint

During mitotic (Hoyt et al., 1991; Li and Murray, 1991) and meiotic (Shonn et al., 2000) cell division, the SAC ensures that chromosome segregation does not occur before all chromosomes are captured on a bipolar spindle. Normally in mitosis of budding yeast, the time required for formation of a bipolar mitotic spindle and chromosome attachment to the spindle appears to be shorter than the time required to biochemically activate cell cycle machinery for progression to the next stage of the cell cycle (Li and Murray, 1991). However, if spindle formation, sister chromatid capture, or tension across kinetochores is compromised, the SAC is required to prevent premature onset of cell division (Li and Murray, 1991). For example, mutations leading to defects in centromeric DNA (Spencer and Hieter, 1992), kinetochore function (Wang and Burke, 1995), microtubules (Li and Murray, 1991), or microtubule motor function (Hardwick et al., 1999) can activate the SAC. Destabilizing microtubules with drugs also necessitates a SAC arrest to ensure cell survival (Hoyt et al., 1991; Li and Murray, 1991). In the absence of checkpoint function, cells attempt to divide prior to achievement of a fully formed or accurately attached mitotic spindle, leading to dramatic chromosome missegregation (Li and Murray, 1991).

The SAC was identified using screens for mutants which fail to arrest and subsequently lose viability in the presence of the microtubule-destabilizing drug benomyl (Hoyt et al., 1991; Li and Murray, 1991). Thus, mutants identified in these screens were classified as "mitotic arrest deficient" (*mad*) (Li and Murray, 1991) or "budding uninhibited by benzimidazole" (*bub*) (Hoyt et al., 1991) mutants. Characterization of products encoded by these genes provided the foundation for the current understanding of SAC activation, regulation, and its effectors. Components of the SAC in yeast include Mad1p, Mad2p, Mad3p (Li and Murray, 1991), Bub1p, Bub3p (Hoyt et al., 1991), Mps1p (Weiss and Winey, 1996), and Ipl1p (Biggins and Murray, 2001). The SAC arrests cells by inhibiting the anaphase promoting complex (APC) protein Cdc20p. A simplified view of the SAC is that Mad2p cycles on and off of unattached kinetochores, becoming activated. This activated form of Mad2p binds to and inhibits Cdc20p (Hwang et al., 1998). Upon proper bipolar attachment of all chromosomes to the mitotic spindle, Cdc20p is released by Mad2p and can functionally interact with the APC. This facilitates the ubiquitination and degradation of downstream APC targets, eventually leading to sister chromatid separation (Rudner and Murray, 1996; Yamamoto et al., 1996; Straight and Murray, 1997; Fraschini et al., 1999).

This is a simplified view of the checkpoint, since all members of the SAC appear to be required for a fully functional SAC. Additionally, there is some debate as to whether the SAC simply detects unattached kinetochores or whether it also monitors spindle tension at kinetochores (Biggins and Murray, 2001). It appears both mechanisms may contribute to checkpoint monitoring of kinetochore attachment. In budding yeast, the Aurora kinase Ipl1p appears to participate in the tension sensing branch of the SAC (Biggins and Murray, 2001). It has been postulated that Ipl1p responds to a lack of tension generated across kinetochores (for example, upon kinetochore attachment to a monopolar spindle) by disrupting kinetochore-microtubule attachments. This produces unattached kinetochores which are detected by Mad2p (Biggins and Murray, 2001).

Most components of the SAC are conserved in higher eukaryotes, though differences do exist. For example, the Mad3p homologue BubR1 has kinase activity in human cells where it is part of a complex known as the "mitotic checkpoint complex" (MCC) (Sudakin et al., 2001). The MCC also contains hBub1, Cdc20, and Mad2 and is reportedly a much better inhibitor of APC^{Cdc20} than Mad2 alone (Sudakin et al., 2001). Also, in vertebrate cells, the SAC functions in every cell cycle (Gorbsky et al., 1998; Shah and Cleveland, 2000). In contrast, SAC activation in *S. cerevisiae* only occurs in response to specific mitotic spindle defects (Gillett et al., 2004).

1.6.2 The Spindle Position Checkpoint

Functioning later in mitosis in yeast, the spindle position checkpoint ensures that mitotic spindle breakdown and cytokinesis do not occur before spindle elongation into the bud is complete (Pereira et al., 2000). It is activated in response to defects causing delayed spindle delivery to the bud and functions by postponing the activity of the mitotic exit network (MEN). The MEN is a signalling cascade which leads to APC^{Cdh1} activation, degradation of mitotic cyclins (Jaspersen et al., 1998), cytokinesis, and entry into G1-phase of the subsequent cell cycle (Li, 1999). It includes Cdc15p, Lte1p, Mob1p, Dbf2p, Tem1p, Cdc14p, and Cdc5p (Jaspersen et al., 1998; Fesquet et al., 1999; Hu et al., 2001). Conversion of the GTPase Tem1p to its active (GTP bound) form signals for mitotic exit. The GTPase activating complex for Tem1p (Pereira et al., 2000; Lee et al., 2001), maintaining Tem1p in its inactive (GDP bound) state, consists of Bub2p (Fraschini et al., 1999) and Bfa1p (Shirayama et al., 1994). Bub2p/Bfa1p localize preferentially to the bud bound SPB (Li, 1999). Lte1p, the putative Tem1p-GEF (Shirayama et al., 1994), is localized exclusively to the bud cortex prior to late anaphase (Pereira et al., 2000). This establishes a model whereby the Tem1p-GAP activity of Bub2p/Bfa1p maintains Tem1p in its inactive form in the mother cell. In contrast, entry of the SPB into the bud allows Tem1p to encounter the GEF activity of Lte1p and become activated within the bud (Pereira et al., 2000; Lee et al., 2001). Theoretically, this links SPB entry into the bud with MEN signalling (Pereira et al., 2000; Lee et al., 2001). There is debate over this model, however, since deletion of *LTE1* has little effect on the timing of exit from mitosis (Adames et al., 2001). It is proposed that interactions between cytoplasmic microtubules and the bud neck can also signal the MEN (Adames et al., 2001). Furthermore, the kinase Cdc5p may influence Tem1-GAP activity via cell cycle regulated phosphorylation of Bfa1p, which reduces the ability of Bfa1p to interact with its target Tem1p (Hu et al., 2001). This favours the production of Tem1-GTP and exit from mitosis. After the completion of anaphase, dephosphorylation of Bfa1p by the phosphatase Cdc14p may reactivate Bub2p/Bfa1p GAP activity (Pereira et al., 2002). Whatever the mechanism, it is clear that any mutations which delay daughter-bound SPB entry into the bud will consequently delay mitotic exit. Defects in this checkpoint in combination with delayed nuclear migration or spindle positioning can lead to chromosome missegregation (see Schuyler and Pellman, 2001; Lew and Burke, 2003).

Both the SAC and spindle positioning checkpoints monitor microtubule function. Mutations which decrease microtubule organization, stability, dynamics, or orientation require one or both of these checkpoints to prevent chromosome missegregation or genetic instability (Lew and Burke, 2003). Phenotypically, both spindle checkpoint mutants and microtubule-organizing mutants tend to exhibit altered growth in the presence of microtubule-destabilizing drugs such as benomyl or nocodazole (Hoyt et al., 1991; Li and Murray, 1991; Cottingham and Hoyt, 1997; Straight and Murray, 1997; Richards et al., 2000).

1.7 Focus of This Thesis

In an effort to identify new functional relationships between the nuclear transport machinery and cell cycle regulatory components, we screened numerous *S. cerevisiae* nup and kap mutants for altered growth in the presence of benomyl. We identified a benomyl sensitive phenotype for a mutant lacking the β-karyopherin Kap123p. We describe the characterization of the *kap123Δ* null mutant with respect to previously described checkpoint and microtubule mutants and propose a novel role for Kap123p in microtubule stability.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Yeast Strains and Media

Yeast strains used are shown in *Table 2-1*. Yeast strains were grown at 30°C, unless otherwise stated, in YPD (1% yeast extract, 2% bactopeptone, and 2% glucose) or synthetic media (SM) supplemented with the appropriate nutrients and 2% glucose (Sherman et al., 1983). 5-fluoroorotic acid (5-FOA) (Toronto Research Chemicals, Inc., North York, Ontario, Canada) containing plates were prepared as previously described (Boeke et al., 1984). Yeast transformations were performed as previously described (Thompson et al., 1998). Sporulation of diploid strains and tetrad dissections were done as in Adams et al. (1997). All PCR products described were amplified using the Expand High Fidelity PCR System (Roche Diagnosics, Laval, Quebec, Canada) and purified using a QIAquick PCR purification kit (Qiagen, Mississauga, Ontario, Canada).

Table 2-1. Yeast strains.

Strain	**Genotype**	**Source, Derivation, Or Reference**
DF5a	*Mata ura3-52 his3Δ200 trp1-1 leu2-3,112 lys2-801*	
DF5α	*Matα ura3-52 his3Δ200 trp1-1 leu2-3,112 lys2-801*	
KP123	*Matα ura3-52 his3Δ200 trp1-1 leu2-3,112 lys2-801 kap123Δ::URA3*	Rout et al., 1997
KP123-pA	*Matα ura3-52 his3Δ200 trp1-1 leu2-3,112 lys2-801 KAP123-PA-URA3-HIS3*	Rout et al., 1997
YAA3500	*Mata ura3-52 his3Δ200 trp1-1 leu2-3,112 lys2-801 kap123Δ::URA3*	this work
YAA3501	*Mata ura3-52 his3Δ200 trp1-1 leu2-3,112 lys2-801 kap123Δ::KAN*	this work
YMB1904	*Mata ura3-52 lys2-801 his3-200 leu2-3,112 trp1-1 mad2Δ::HIS3*	Iouk et al., 2002
YMB1906	*Matα ura3-52 lys2-801 his3-200 leu2-3,112 trp1-1 mad2Δ::HIS3*	Iouk et al., 2002
YPH277	*Mata ura3-52 lys2-801 ade2-101 trp1Δ1 leu2Δ1 CFVII(RAD2d.YPH277)URA3 SUP11*	Spencer et al., 2000; Kerscher et al., 2001

Strain	**Genotype**	**Source, Derivation, or Reference**
YAA3508	*Mata ura3-52 lys2-801 ade2-101 trp1Δ1 leu2Δ1 CFVII(RAD2d.YPH277)URA3 SUP11 kap123Δ::KAN*	this work
YPH278	*Matα ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 CFIII (CEN3L.YPH278)URA3 SUP11*	Spencer et al., 2000; Kerscher et al., 2001
YAA3509	*Matα ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 CFIII (CEN3L.YPH278)URA3 SUP11 kap123Δ::KAN*	this work
DBY2411	*Mata his3Δ200 leu2-3, 112 lys2-801 ura3-52 tub1Δ::HIS3 tub3Δ::TRP1 tub1-729-LEU2-CEN4-ARS1*	Schatz et al., 1988
YAA3528	*Mata his3Δ200 leu2-3, 112 lys2-801 ura3-52 tub1Δ::HIS3 tub3Δ::TRP1 tub1-729-LEU2-CEN4-ARS1 kap123Δ::URA3*	this work
YAA3502	*Matα ura3-52 lys2-801 his3-200 leu2-3,112 trp1-1 mad2Δ::HIS3 kap123Δ::URA3*	this work
YAA3503	*Mata his3Δ200 trp1-1 leu2-3,112 lys2-801 ura3-52:: URA3-GFP-TUB1*	DF5a transformed with the integrating plasmid pAFS92-*URA3-GFP-TUB1* (Adames and Cooper, 2000)
YAA3504	*Mata his3Δ200 trp1-1 leu2-3,112 lys2-801, ura3-52::URA3-GFP-TUB1 kap123Δ::KAN*	YAA3501 transformed with the integrating plasmid pAFS92-*URA3-GFP-TUB1* (Adames and Cooper, 2000)
M1GFP	*Matα ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 MAD1-GFP-HIS5*	Iouk et al., 2002
YAA3505	*Matα ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 MAD1-GFP-HIS5 kap123Δ::URA3*	this work
BY4742	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Research Genetics
BY4741	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
yer110Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 kap123Δ::KAN*	Research Genetics
YAA3506	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 kap123Δ::HIS5*	this work
yjl030Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mad2Δ::KAN*	Research Genetics
YAA3507	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mad2Δ::HIS3*	this work
ygl216Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 kip3Δ::KAN*	Research Genetics
ynl271Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 bni1Δ::KAN*	Research Genetics
ygl216Δ/ YAA3507	*Mata/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 MAD2/mad2Δ::HIS3 KIP3/kip3Δ::KAN*	diploid produced by mating of ygl216Δ and YAA3507
YAA3520	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 kip3Δ::KAN mad2Δ::HIS3*	segregant from sporulation and dissection of ygl216Δ/YAA3507
ynl271Δ/ YAA3507	*Mata/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 MAD2/mad2Δ::HIS3 BNI1/bni1Δ::KAN*	diploid produced by mating of ynl271Δ and YAA3507
YAA3521	*Matα his3Δ1 leu2Δ0 ura3Δ0 bni1Δ::KAN mad2Δ::HIS3*	segregant from sporulation and dissection of ynl271Δ/YAA3507
ygl241Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 kap114Δ::KAN*	Research Genetics
ygl216Δ/ YAA3506	*Mata/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 ura3Δ0/ura3Δ0 LYS2/lys2Δ0 KAP123/ kap123Δ::HIS5 KIP3/kip3Δ::KAN*	diploid produced by mating of YAA3506 and ygl216Δ

Strain	Genotype	Source, Derivation, or Reference
YAA3522	*Mata his3Δ1 leu2Δ0 ura3Δ0 kip3Δ::KAN kap123Δ::HIS5*	segregant from sporulation and dissection of YAA3506/ygl216Δ
ynl271Δ/ YAA3506	*Mata/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 ura3Δ0/ura3Δ0 LYS2/lys2Δ0 KAP123/kap123Δ::HIS5 BNI1/bni1Δ::KAN*	diploid produced by mating of YAA3506 and ynl271Δ
YAA3523	*Mata his3Δ1 leu2Δ0 ura3Δ0 bni1Δ::KAN kap123Δ::HIS5*	segregant from sporulation and dissection of ynl271Δ/YAA3506
yjl187Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 swe1Δ::KAN*	Research Genetics
yjl187Δ/ YAA3506	*Mata/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 MET15/met15Δ0 ura3Δ0/ura3Δ0 KAP123/kap123Δ::HIS5 SWE1/swe1Δ::KAN*	diploid produced by mating of YAA3506 and yjl187Δ
YAA3524	*Mata his3Δ1 leu2Δ0 ura3Δ0 swe1Δ::KAN kap123Δ::HIS5*	segregant from sporulation and dissection of yjl187Δ/YAA3506
ymr055Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 bub2Δ::KAN*	Research Genetics
ymr055Δ/ YAA3506	*Mata/α his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 KAP123/kap123Δ::HIS5 BUB2/bub2Δ::KAN*	diploid produced by mating of YAA3506 and ymr055Δ
YAA3525	*Mata his3Δ1 leu2Δ0 ura3Δ0 bub2Δ::KAN kap123Δ::HIS5*	segregant from sporulation and dissection of ymr055Δ/YAA3506
yal024Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 lte1Δ::KAN*	Research Genetics
yal024Δ/ YAA3506	*Mata/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 KAP123/kap123Δ::HIS5 LTE1/lte1Δ::KAN*	diploid produced by mating of YAA3506 and yal024Δ
YAA3526	*Matα his3Δ1 leu2Δ0 ura3Δ0 lte1Δ::KAN kap123Δ::HIS5*	segregant from sporulation and dissection of yal024Δ/YAA3506
yml124Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tub3Δ::KAN*	Research Genetics
yml124Δ/ YAA3506	*Mata/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 KAP123/kap123Δ::HIS5 TUB3/tub3Δ::KAN*	diploid produced by mating of YAA3506 and yml124Δ
YAA3529	*Mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	segregant from sporulation and dissection of yml124Δ/YAA3506
YAA3530	*Matα his3Δ1 leu2Δ0 ura3Δ0 kap123Δ::HIS5*	segregant from sporulation and dissection of yml124Δ/YAA3506
YAA3531	*Matα his3Δ1 leu2Δ0 ura3Δ0 tub3Δ::KAN*	segregant from sporulation and dissection of yml124Δ/YAA3506
YAA3527	*Mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tub3Δ::KAN kap123Δ::HIS5*	segregant from sporulation and dissection of yml124Δ/YAA3506
yml094Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gim5Δ::KAN*	Research Genetics

The *URA3* open reading frame and sequence up- and downstream of the *KAP123* locus was amplified from genomic DNA isolated from KP123 (Rout et al., 1997) using the primers 5' - TGGAAAGCGCGATGAGATGA-3' and 5'-GCCGATCAACTTTACAG GAC - 3'. This PCR product was transformed into DF5a to produce YAA3500. The same PCR product was also transformed into YMB1904 to produce YAA3502, into M1GFP to produce YAA3505 and into DBY2411 to produce YAA3528. YAA3501 was constructed using a similar strategy. The *KanMX* open reading frame as well as sequence up- and downstream of the *KAP123* locus was amplified from genomic DNA isolated from yer110Δ using the primers 5' – TCACTTTTCTCATTGCGCTTG - 3' and 5'- TTCTGGGATATTCGCCGAAA - 3'. This PCR product was transformed into the *KAP123* locus of DF5a to produce YAA3501. The same PCR product was transformed into YPH277 and YPH278 strains to produce YAA3508 and YAA3509, respectively. YAA3506 was constructed by integration of a PCR product containing the *S. pombe his5+* open reading frame into the *KAP123* locus of BY4741 cells. This PCR product was obtained using the primers 5'- AAAAGAATAGGCAAGACCCCGCAAGG ATCCTTCTTCTCGTTTCTGTATATTACCAAAATCCGGATCCCCGGGTTAATTAA – 3' and 5' – TGGGAAAGTAAATTTACATATAGTTTTTCTAGGAAAAAAATA AAAATAATGAAGGAAGGGAATTCGAGCTCGTTTAAAC – 3' and the vector pFA6a-His3MX6 (Longtine et al., 1998) as a PCR template. New *kap123Δ* null mutant strains were tested by diagnostic PCR and for benomyl sensitivity. YAA3507 was constructed by integration of a PCR product containing the *HIS3* open reading frame into the *MAD2* locus of BY4741. This PCR product was obtained by amplifying the *HIS3* open reading frame, along with 600 bp up- and downstream of the *MAD2* locus, from genomic DNA isolated from YMB1904 using the primers 5'-GACCCAAAGTCTGTG ATTCCA - 3' and 5' – AGGGTGAAAAGACATGCAGA - 3'. Transformants were tested for *mad2Δ* deletion by plating serial dilutions of transformant cells on benomyl-containing plates to determine benomyl sensitivity relative to wild type. As well, transformant cells were tested for expression of Mad2p by separation of whole cell lysates by SDS-PAGE, followed by Western blotting using rabbit polyclonal antibodies directed against Mad2p (R.J. Scott, unpublished). The cold sensitive tubulin mutant DBY2411 (Schatz et al., 1988) was a generous gift from Dr. Frank Solomon (Massachusetts Institute of Technology, Cambridge, MA). YAA3503 and YAA3504, which contain *GFP-TUB1* under the control of the *MET3* promoter, were constructed by transformation of DF5a and YAA3501, respectively, with the *StuI* digested plasmid pAFS92-*URA3-GFP-TUB1* (Dr. Neil Adames, University of Alberta, Edmonton, AB) which integrates at the *ura3* locus (Adames and Cooper, 2000). As indicated in *Table 2-1*, YAA3506 was mated to a number of yeast deletion mutants to generate diploid strains. Prior to mating, YAA3506 was transformed with the plasmid pYEX-*KAP123* to cover the *kap123Δ* deletion. After sporulation and dissection of the diploid strains, resultant haploid cells were cured of the *URA3* containing pYEX-*KAP123* plasmid by plating strains on 5-FOA containing plates.

2.2 Plasmids

2.2.1 Parent Plasmids

Parent plasmids used in this work were: pRS315 *CEN/LEU2* (Sikorski and Hieter, 1989), pRS316 *CEN/URA3* (Sikorski and Hieter, 1989), pRS317 *CEN/LYS2* (Sikorski and Hieter, 1989), and pYEX BX (BD Biosciences Clontech, San Jose, CA).

2.2.2 Derived Plasmids

To generate a PCR product containing the *KAP123* open reading with *BglII* digestible ends, genomic DNA was isolated from DF5a and the *KAP123* open reading frame was amplified from this genomic DNA using the primers 5'-GCAGCAGATCTATGGAT CAACAATTTCTA-3' and 5' - GCAGCAGATCTTCAAGCAATGACGGCAGC-3'. This PCR product was digested with the restriction enzyme *BglII*. pYEX-*KAP123* was constructed by ligating the digested PCR product into *BamHI* digested pYEX BX (BD Biosciences Clontech, San Jose, CA). The *KAP121* overexpressing plasmid pYEX-*KAP121-PA* was constructed by ligating the *KAP121* open reading frame fused to cDNA coding for protein A (pA) into the pYEX BX plasmid (BD Biosciences Clontech, San Jose, CA) (C.P. Lusk, unpublished). pRS317-*KAP123* was constructed by ligating the *KAP123* open reading frame into pRS317 *CEN/LYS2* (Sikorski and Hieter, 1989) (D. Leslie, unpublished). pAFS92-*URA3-GFP-TUB1* was a kind gift from Dr. Neil Adames (University of Alberta, Edmonton, AB).

2.3 Drug Treatments

Unless otherwise stated, nocodazole (Sigma Aldrich, Oakville, Ontario, Canada) was used at a concentration of 15 μg/ml, and α-factor (Sigma Aldrich, Oakville, Ontario, Canada) was used at a concentration of 7 μg/ml. Benomyl (Sigma Aldrich, Oakville, Ontario, Canada) was used at concentrations indicated and was added to plates as previously described (Hyland et al., 1999; Iouk et al., 2002). The microtubule-destabilizing drugs benomyl and nocodazole were used interchangeably for experiments in which microtubules were depolymerised using drug treatment. The more cost efficient drug benomyl was used to destabilize the microtubules cells grown on solid medium. Nocodazole was used for experiments performed in liquid medium due to the low solubility of benomyl.

2.4 Viability Assay

Logarithmically growing yeast cultures were treated with nocodazole (15 μg/ml) and incubated at 30°C. At 0, 3 and 6 h after addition of the drug, individual cells were manipulated into a grid pattern on YPD plates using a dissecting microscope. Plates were incubated at 30°C for 48 h. Viability in nocodazole was calculated by dividing the number of colonies formed after 0, 3, or 6 h in nocodazole by the number of colonies formed at time 0 (Straight and Murray, 1997). 50 cells were plated per strain per time point; n=3 or 5, as indicated.

2.5 FACS analysis

FACS analysis was performed as previously described (Makhnevych et al., 2003). DNA was stained with propidium iodide (Sigma Aldrich, Oakville, Ontario, Canada) and detected with a FACScan (Becton Dickinson, San Jose, CA) flow cytometer. Data was analyzed using CellQuest software (BD Biosciences Clontech, San Jose, CA).

2.6 Chromosome loss assay

The *KAP123* open reading frame was replaced with *URA3* in YPH277 and YPH278 (Spencer et al., 1990), as described, to produce YAA3508 and YAA3509. Serial dilutions of wild type and *kap123Δ* null mutants from YPH277 and YPH278 parental backgrounds were plated onto YPD and incubated at 16°C, 23°C, 30°C or 37°C for 48 h or onto YPD containing vehicle (DMSO) or vehicle plus increasing concentrations of benomyl and incubated at 30°C for 48 h. Colonies were monitored for red colour development or colony sectoring indicative of chromosome missegregation (see section 3.2.2).

2.7 Fluorescence Microscopy

GFP fusion proteins were visualized on an Olympus BX50 fluorescence microscope equipped with a SPOT digital camera (Diagnostics Instruments, Inc., Sterling Heights, MI). Cells were taken from exponentially growing cultures. For the visualization of GFP-tagged α-tubulin, strains containing integrated pAFS92-*URA3-GFP-TUB1* were grown in CM-Met liquid medium for 2 h at 30°C to induce expression of *GFP-TUB1*. In experiments in which GFP-Tub1p expressing cells were synchronized in G1-phase, cultures were transferred from CM-Met to complete liquid medium following induction. α-factor was added and cultures were incubated at 30°C for ~2 h until arrested in G1-phase, as determined by examination of cells by light microscopy. To remove α-factor and release cells from G1-phase arrest, cells were washed twice with pre-warmed (30°C) YPD. DNA was stained by adding 1 μl of 10 mM Hoechst to 1 ml of cells resuspended in PBS. After 1 min of staining, excess Hoechst was removed by washing cells twice with PBS.

2.8 Affinity Precipitation

Affinity precipitations were performed as previously described (Lusk et al., 2002). 500 ml cultures of a strain expressing protein A tagged Kap123p (KP123-pA) (Rout et al., 1997) or an untagged control (DF5a) were grown to an OD_{600} of 0.6. Cells were harvested by centrifugation, washed, and resuspended in 15 ml lysis buffer (20 mM Na_2HPO_4, pH 7.5, 150 mM NaCl, 0.1 mM $MgCl_2$,). Cells were lysed with a French Press before adding an equal volume of lysis buffer containing 40% DMSO and 2% Triton X-100. Lysates were cleared by centrifugation at 11,300 x g, followed by centrifugation at 311,000 x g. The resulting supernatant was incubated for 90 min with pre-equilibrated IgG beads (Amersham Biosciences Inc., Baie D'Urfe, Quebec, Canada) at 4°C. Beads were washed with buffer containing 20 mM Na_2HPO_4, pH 7.5, 150 mM NaCl, 0.1 mM $MgCl_2$, and 0.1% Tween 20. Proteins were eluted from the beads using increasing concentrations of $MgCl_2$. The final elution was done with 0.5 M CH_3COOH, pH 3-4. Proteins were analyzed by Western blotting.

2.9 SDS-PAGE and Western blot analysis

Blots for tubulin proteins were performed as follows. Proteins from yeast whole cell lysates were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Post-transfer nitrocellulose membranes were blocked with 1.5% gelatin/1% BSA in TBS containing 0.1% Tween-20 (TBS/T-20). Tub4p was detected using specific, affinity purified, rabbit polyclonal antibodies (Dr. Jackie Vogel (McGill, Montreal, Quebec, Canada)) (Vogel and Snyder, 2000). Tub1p was detected using a rat monoclonal antibody (YOL 1/34) directed against yeast α-tubulin (Accurate Chemical & Scientific

Corporation, Westbury, NY). Anti-Tub4p and anti-Tub1p primary antibodies were used at dilutions of 1:3000 and 1:300, respectively. Primary antibodies were incubated with membranes overnight at 4°C. Membranes were washed with TBS/T-20. For anti-Tub4p blots, secondary HRP-conjugated, donkey anti-rabbit antibody (Amersham Biosciences Inc., Baie D'Urfe, Quebec, Canada) was used at a dilution of 1:2000 for 2 h at ambient temperature. For anti-Tub1p blots, the secondary HRP-conjugated, rabbit anti-rat antibody STAR21B (Serotec, Inc., Raleigh, NC) was used at a dilution of 1:2000 for 2 h at ambient temperature. Membranes were washed as before and protein/antibody complexes visualized with ECL reagent as described by the manufacturer (Amersham Biosciences Inc., Baie D'Urfe, Quebec, Canada). Other blots were performed as previously described (Lusk et al., 2002). Post-transfer nitrocellulose membranes were blocked with 5% skim milk powder in PBS containing 0.1% Tween 20 (PBS/T-20). Gsp1p (Makhnevych et al., 2003), Mad2p (R.J. Scott, unpublished), GFP (R.J. Scott, submitted manuscript), and Clb2p (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were detected using specific rabbit polyclonal antibodies. Membranes were washed using PBS/T-20. Secondary antibodies used were HRP-conjugated, donkey anti–rabbit (Amersham Biosciences Inc., Baie D'Urfe, Quebec, Canada). Protein A was detected using rabbit affinity purified antibody raised against mouse IgG (ICN/Cappel, Aurora, OH) or with secondary HRP-conjugated, donkey anti–rabbit antibody alone (Amersham Biosciences Inc., Baie D'Urfe, Quebec, Canada). Proteins were visualized using ECL as described by the manufacturer (Amersham Biosciences Inc., Baie D'Urfe, Quebec, Canada).

2.10 Interactions Network

Genetic interaction and affinity precipitation/mass spectrometry data for Kap123p obtained from this work were combined with reported genetic and physical interactions of Tub1p, Tub3p, Tub4p, and Gim5p (see *Table 3-2*). Overlapping interactions between these proteins were graphed using Cytoscape 2.0 Software (www.cytoscape.org) (Shannon et al., 2003) with manual clustering of nodes. Each node represents an individual protein. Genetic interactions are designated by a pink connection (edge) and predicted physical interactions are designated by a blue connection (edge).

CHAPTER THREE: RESULTS

3.1 *kap123Δ* mutants are sensitive to microtubule-destabilizing drugs

It was previously demonstrated that yeast mutants null for the nucleoporins *NUP53*, *NUP59*, *NUP157*, and *NUP170* are resistant to the microtubule-destabilizing drug benomyl (Iouk et al., 2002). Although the exact mechanism underlying this resistance is yet to be identified, these nups belong to a NPC sub-complex which physically interacts with the SAC protein Mad1p (Iouk et al., 2002). In an effort to identify new relationships between the nuclear transport system and chromosome segregation machinery/cell cycle checkpoints, we screened a variety of kap and nup mutants in yeast for benomyl sensitivity or resistance (R.J. Scott and R.W. Wozniak, unpublished). By this method, we observed that a mutant lacking the non-essential β-karyopherin Kap123p is sensitive to the microtubule-destabilizing drug benomyl (*Figure 3-1A*). This benomyl sensitivity can be rescued by transformation of *kap123Δ* cells with a plasmid-borne copy of *KAP123* (*Figure 3-1A*).

To determine the specificity of this benomyl sensitivity, we assessed the growth of other kap mutants on medium containing benomyl. As shown in *Figure 3-1B*, *kap114Δ* cells exhibit wild type growth on benomyl-containing plates. This suggests that cells lacking Kap114p can respond normally to benomyl-induced microtubule disruption and signifies that the benomyl sensitivity observed in *kap123Δ* cells is not a general phenotype of kap mutants. Furthermore, mutants of the β-kaps *kap104*, *msn5*, *los1*, *mtr10*, and *cse1* grow

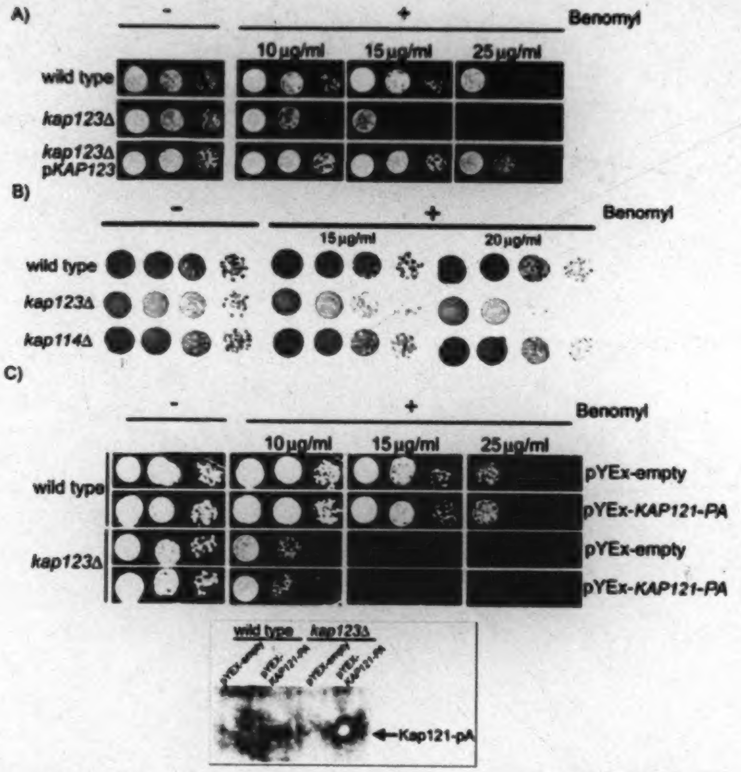

A)
-
+
Benomyl
10 µg/ml
15 µg/ml
25 µg/ml
wild type
kap123Δ
kap123Δ pKAP123
B)
-
+
Benomyl
15 µg/ml
20 µg/ml
wild type
kap123Δ
kap114Δ
C)
-
+
Benomyl
10 µg/ml
15 µg/ml
25 µg/ml
wild type
kap123Δ
pYEx-empty
pYEx-KAP121-PA
pYEx-empty
pYEx-KAP121-PA
wild type
kap123Δ
pYEx-empty
pYEx-KAP121-PA
pYEx-empty
pYEx-KAP121-PA
Kap121-pA

Figure 3-1. Sensitivity of kap123Δ mutant cells to microtubule-destabilizing drugs. **Panel A:** *kap123Δ* cells exhibit decreased growth in the presence of the microtubule-destabilizing drug benomyl. Serial dilutions of wild type (DF5a) cells, *kap123Δ* (YAA3500) cells, or *kap123Δ* cells (YAA3500) containing plasmid-borne *KAP123* were spotted onto YPD plates containing vehicle alone (DMSO) (-) or vehicle plus increasing concentrations of benomyl (+) and incubated at 30°C for 48 h. **Panel B:** Benomyl sensitivity is not a general defect of *kap* mutants. To determine the specificity of the benomyl sensitivity observed for *kap123Δ* cells (yer110Δ), this mutant was plated in parallel with wild type cells (BY4742) as well as with mutant cells lacking the β-kap Kap114p (ygl241Δ) on YPD (-) or YPD containing benomyl (+), as in panel A. **Panel C:** Over-expression of the β-kap Kap121p does not rescue the benomyl sensitivity of *kap123Δ* cells. Serial dilutions of wild type (BY4742) and *kap123Δ* (yer110Δ) cells, transformed with an empty pYEX plasmid or with pYEX-*KAP121-PA*, were spotted on YPD (-) or YPD containing benomyl (+), as above. Overexpression of Kap121-pA was analyzed by separation of total cell lysates from wild type cells (BY4742) and *kap123Δ* cells (yer110Δ), containing an empty vector (pYEX) or pYEX-*KAP121-PA*, by SDS-PAGE followed by Western blotting for protein A with HRP-conjugated donkey anti-rabbit antibody.

as wild type in the presence of benomyl (R.J. Scott and R.W. Wozniak, unpublished). Taken together, these data indicate that the benomyl sensitivity of *kap123Δ* cells is caused by the loss of a specific function of Kap123p.

We were interested in determining whether another kap can functionally substitute for Kap123p in the pathway influencing the benomyl sensitivity of cells. The related β-kap Kap121p/Pse1p seemed the most likely candidate for replacing Kap123p. Kap123p shares approximately 20% identity and 50% similarity (Rout et al., 1997) with Kap121p. These two β-kaps share the highest degree of structural similarity among β-kap family members (Wozniak et al., 1998). Consistent with this, it was previously shown that overexpression of Kap121p can rescue much of the ribosomal protein import, normally performed by Kap123p, in a *kap123Δ* mutant (Rout et al., 1997), suggesting there is significant overlap in the repertoire of cargoes they import. We speculated that if the loss of an import function is related to the benomyl sensitivity of the *kap123Δ* strain, perhaps overexpression of *KAP121* might rescue the growth of *kap123Δ* cells in the presence of benomyl. To assess whether overexpression of *KAP121* in *kap123Δ* cells can improve growth on benomyl-containing media, we transformed wild type and *kap123Δ* cells with an empty pYEX plasmid or with pYEX-*KAP121-PA* and plated the strains on benomyl-containing media. Increasing the levels of Kap121p-pA does not improve the growth of *kap123Δ* cells in the presence of benomyl (*Figure 3-1C*). We concluded from these data that benomyl sensitivity linked to the loss of Kap123p function is unique and non-overlapping with Kap121p.

Having determined that benomyl sensitivity is a distinctive trait of *kap123Δ* cells, we attempted to identify the functional cause of this drug effect. To do this, we characterized the *kap123Δ* mutant based on phenotypes associated with mutations known to result in benomyl sensitivity. Sensitivity of yeast strains to microtubule-destabilizing drugs is a phenotype commonly associated with mutants defective in the SAC (Hoyt et al., 1991; Li and Murray, 1991) or in tubulin folding, microtubule organization (Hoyt et al., 1997; Geissler et al., 1998), stability (Schatz et al., 1988; Hoyt et al., 1997; Richards et al., 2000), or dynamics (Cottingham and Hoyt, 1997).

Though mutants defective in SAC or microtubule function share a characteristic benomyl sensitivity, the underlying cause of the drug sensitivity is distinct for each group of mutants. The benomyl sensitivity of SAC mutants reflects a failure to respond appropriately to microtubule disruption. The SAC monitors attachment of chromosomes to the mitotic spindle, arresting cells at the metaphase to anaphase transition in response to misaligned or unattached chromosomes (Hoyt et al., 1991; Li and Murray, 1991). Destabilizing microtubules with drugs (i.e. nocodazole or benomyl) necessitates a SAC response to prevent chromosome missegregation. In the absence of a functional SAC, cells do not arrest in response to these drugs and, thus, missegregate chromosomes which results in a rapid loss of cell viability (Straight and Murray, 1997).

In contrast to SAC mutants, microtubule mutants are competent to elicit a checkpoint response to microtubule disruption. However, because they have a decreased ability to recover from drug-induced microtubule disruption, these mutants require longer checkpoint arrests. SAC activity must be sustained for an extended time to allow microtubules to reorganize and to establish chromosome attachment to the mitotic spindle. This leads to longer checkpoint arrests and slower growth. In the case of severe microtubule defects, cells are never capable of assembling microtubules in the presence of microtubule drugs and eventually die (Straight and Murray, 1997).

We hypothesized that the reduced growth of *kap123Δ* cells in benomyl-containing medium could reflect lesions to a SAC or microtubule-related pathway in the absence of Kap123p. To distinguish between these possibilities, we performed a number of experiments designed to assess whether the SAC is fully functional in *kap123Δ* mutant cells, including 1) detecting cellular DNA content before and after treatment with nocodazole using FACS analysis, 2) assessing the viability of *kap123Δ* cells following treatment with nocodazole, and 3) assessing the response of cycling mitotic regulators to microtubule disruption in *kap123Δ* cells.

3.2 Mutants lacking Kap123p have a functional spindle assembly checkpoint

3.2.1 *kap123Δ* cells exhibit a mitotic delay in response to nocodazole-induced microtubule disruption

The SAC arrests cells at the metaphase to anaphase transition. At metaphase, haploid strains of *S. cerevisiae* have a 2n DNA complement since cells have replicated DNA in S-phase but have not yet divided. Because the addition of nocodazole to yeast cultures disrupts microtubules, cells must respond with a SAC-mediated arrest to avoid premature cell division in the absence of spindle-mediated separation of sister chromatids. Cells with a functional SAC arrest with 2n DNA content that can be detected by FACS analysis. Conversely, when mutants defective for SAC function are treated with nocodazole, cells cannot maintain a SAC arrest and attempt to segregate DNA in the absence of a properly formed mitotic spindle, producing cells with a greater than 2n DNA content (Hoyt et al., 1991; Straight and Murray, 1997). We used FACS analysis to assess the DNA content of asynchronous versus nocodazole treated cultures of wild type, *kap123Δ* and a SAC mutant, *mad2Δ* cells. Wild type cells display a markedly increased 2n DNA peak 2 h after nocodazole addition (*Figure 3-2A*). In contrast, as has previously been shown (Hoyt et al., 1991; Straight and Murray, 1997), the addition of nocodazole to *mad2Δ* cultures does not result in an accumulation of cells with 2n DNA (*Figure 3-2A*). Rather, these cells proceed through mitosis and produce cells with a greater than 2n DNA content, represented by a third peak on the FACS profile (*Figure 3-2A*). Similar to wild type cells, nocodazole treatment of *kap123Δ* cells increases the 2n DNA peak relative to that of asynchronously growing cells (*Figure 3-2A*). These data suggest *kap123Δ* cells are capable of responding to spindle disruption with a mitotic delay.

The increase in the 2n DNA peak for *kap123Δ* cultures in nocodazole supports the idea that these cells have a functional SAC. Nevertheless, FACS profiles of asynchronously growing *kap123Δ* cultures exhibit an additional, greater than 2n, DNA peak not present in wild type cultures (*Figure 3-2A*). This suggests that a population of *kap123Δ* cells is aneuploid. The SAC mutant *mad2Δ* cultures also accumulate cells with an aneuploid

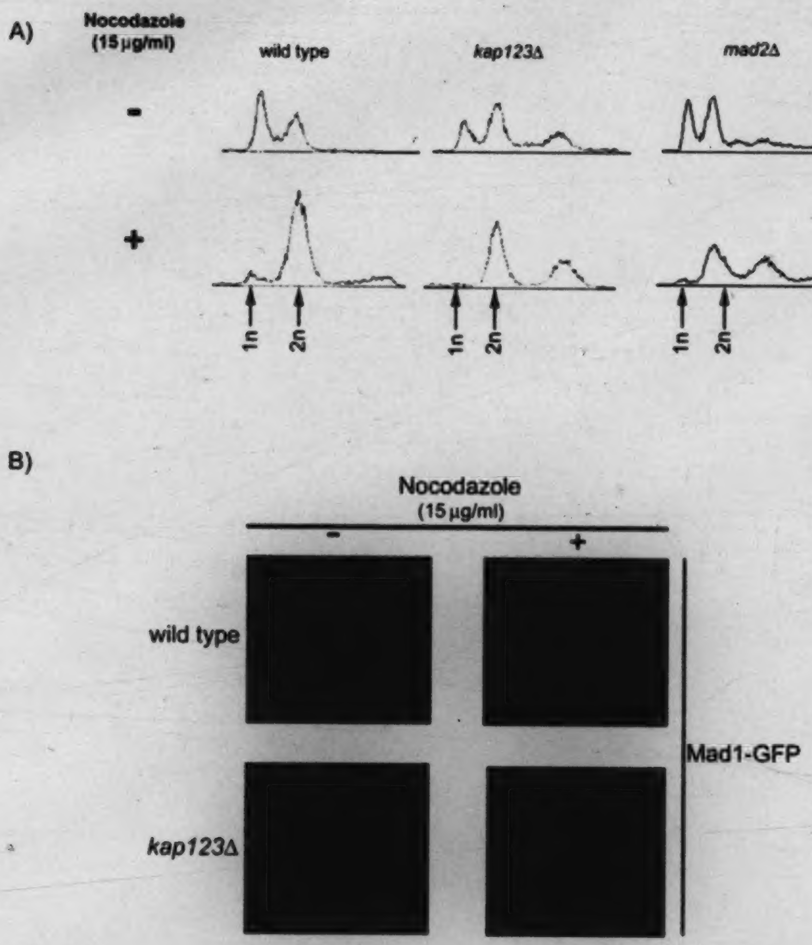


Figure 3-2. kap123Δ cells exhibit a G2/M-phase delay in response to microtubule disruption. **Panel A:** *kap123Δ* cells arrest in G2/M-phase following treatment with nocodazole. FACS analysis was performed on wild type (DF5a), *kap123Δ* (YAA3500), or *mad2Δ* (YMB1906) strains to measure DNA content in the presence or absence of nocodazole-induced microtubule disruption. Logarithmically growing cells were treated with vehicle alone (DMSO) (-) or vehicle containing 15 µg/ml nocodazole (+) and incubated at 30°C for 2 h before preparation of cells for FACS analysis. The positions of peaks representing 1n and 2n DNA are indicated. **Panel B:** The SAC protein Mad1p exhibits normal localization in *kap123Δ* cells growing asynchronously or in the presence of nocodazole. The parental (M1GFP) and *kap123Δ* (YAA3505) strains producing Mad1-GFP were grown to logarithmic phase in complete liquid medium and treated with vehicle alone (DMSO) (-) or vehicle containing nocodazole (+). Cells were examined following 3 h of incubation at 30°C by fluorescence microscopy.

DNA peak, though only after addition of nocodazole (Fraschini et al., 1999) (*Figure 3-2A*). The aneuploid DNA peak present in *kap123Δ* cultures does increase slightly following nocodazole addition (*Figure 3-2A*). However, based on our data, we speculate that it does not reflect an inability of *kap123Δ* cells to arrest in mitosis. Rather, a population of cells appear to have a constitutive aneuploidy in the absence of Kap123p which may be exacerbated in the presence of microtubule destabilizing drugs (see section 3.3.5).

Overall, our FACS data are consistent with the conclusion that *kap123Δ* cells possess a functional SAC. In agreement with this, our data also suggest Kap123p is unlikely to play a role in the import of the SAC protein Mad1p. We tested the effect of the *kap123Δ* mutation on the localization of Mad1p. Mad1p and Mad2p play a critical role in the SAC and are associated with the NPC during interphase (Iouk et al., 2002). From there, they are recruited to kinetochores when the SAC is activated (Iouk et al., 2002; Gillett et al., 2004). As shown in *Figure 3-2B*, the peripheral nuclear localization of a GFP-tagged version of Mad1p is not altered in a *kap123Δ* strain both in asynchronous and nocodazole-treated, M-phase arrested cells. These data argue against a role for Kap123p in the nuclear import of Mad1p.

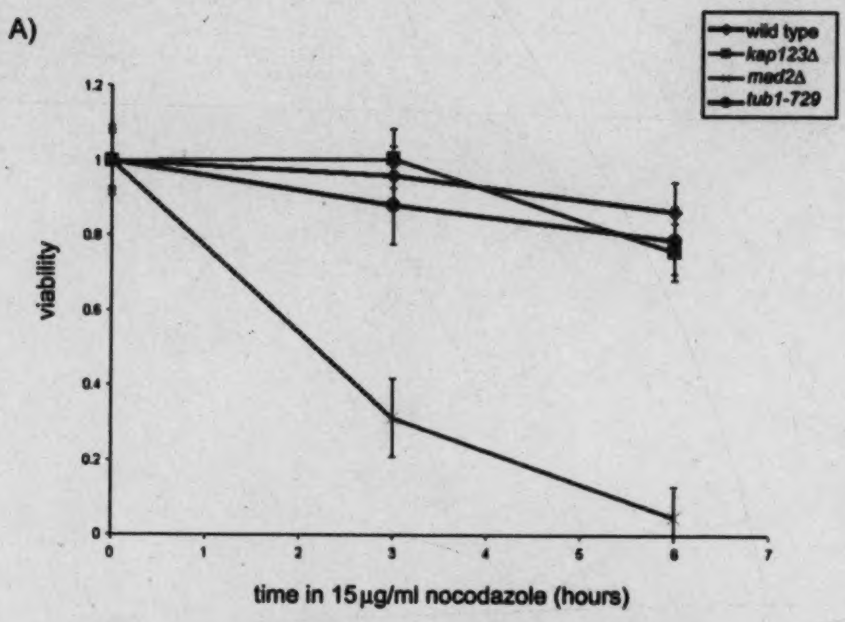
3.2.2 Cells lacking Kap123p remain viable following treatment with nocodazole

A characteristic feature of cells that possess a defect in the SAC is a loss of viability resulting from nocodazole-induced microtubule disruption (Straight and Murray, 1997). This cell death is a consequence of gross chromosome missegregation resulting from premature cell division in the absence of a mitotic spindle. In contrast, cells which are defective in microtubule function arrest in and remain viable following exposure to nocodazole, only dying upon long exposures if microtubule damage is too severe (Straight and Murray, 1997). We hypothesized that if *kap123Δ* cells are defective for SAC function, treatment of *kap123Δ* cultures with nocodazole will result in rapid viability loss whereas if the defect is linked to tubulin functionality, *kap123Δ* cells will arrest and remain viable. To test this, we exposed wild type cells, *kap123Δ* cells, tubulin mutants and SAC mutants to nocodazole for up to 6 h and then tested their ability to grow in complete media. Cultures growing in complete liquid medium were treated with nocodazole and sampled 0, 3, and 6 h following nocodazole addition (*Figure 3-3A*). Viability in nocodazole was calculated by dividing the number of colonies formed after 0, 3, or 6 h in nocodazole by the number of colonies formed at time 0 (Straight and Murray, 1997). While cells lacking the SAC protein Mad2p (*mad2*Δ) rapidly lose viability in nocodazole, *kap123Δ* cells, like wild type cells and tubulin mutant *tub1-729* cells, remain viable after 6 h in nocodazole. These data further support the assertion that the sensitivity of *kap123Δ* cells to microtubule-destabilizing drugs is not caused by a SAC defect but rather altered microtubule function (see below).

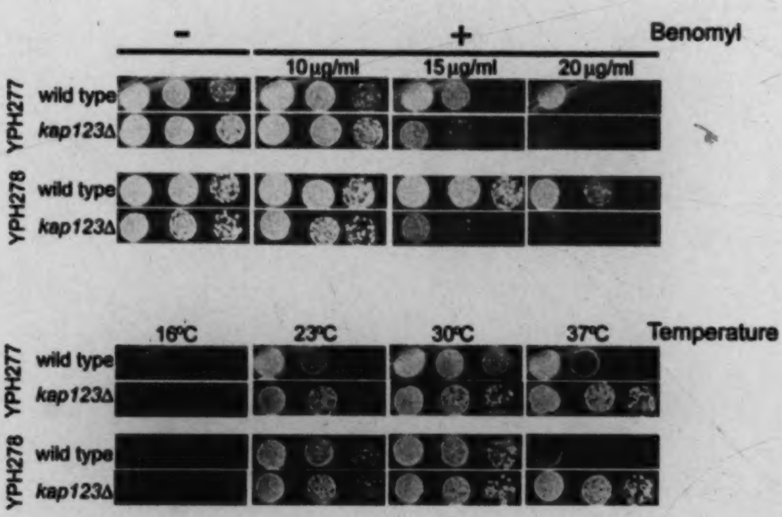
SAC mutants are more prone to chromosome loss than wild type cells, even in the absence of microtubule defects (Li and Murray, 1991). This chromosome loss phenotype is exacerbated following microtubule damage, consistent with the dramatic loss of

Figure 3-3. kap123Δ cells remain viable in nocodazole and do not show gross chromosome loss. **Panel A:** Viability in nocodazole was assessed for wild type (DF5a), *kap123Δ* (YAA3500), *mad2Δ* (YMB1906), or *tub1-729* (DBY2411) strains. Exponentially growing liquid cultures were treated with 15 µg/ml nocodazole at 30°C. Individual cells were isolated from liquid cultures at 0, 3, and 6 h after addition of nocodazole and manipulated into a grid pattern on YPD plates with a dissecting microscope. Plates were incubated at 30°C for 48 h. 50 cells were plated for each strain at each time point; n=3. Viability in nocodazole was calculated by dividing the number of colonies formed after 0, 3, or 6 h in nocodazole by the number of colonies formed at time 0. **Panel B:** *kap123Δ* cells do not exhibit chromosome loss in the presence of benomyl-induced microtubule disruption or upon incubation at temperatures between 16°C and 37°C. Serial dilutions of parental (YPH277, YPH278) and *kap123Δ* (YAA3508, YAA3509) strains were plated onto medium containing vehicle alone (DMSO) (-) or vehicle plus increasing concentrations of benomyl (+). Plates were incubated at 30°C for 48 h (top left panel). Alternately, serial dilutions of wild type (YPH277), *kap123Δ* (YAA3508), wild type (YPH278) and *kap123Δ* (YAA3509) were plated on complete medium and incubated at temperatures between 16°C and 37°C (bottom left panel). Plates were incubated for 48 h.

A)


wild type
kap123Δ
mad2Δ
tub1-729
viability
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
time in 15μg/ml nocodazole (hours)

B)


-
+
Benomyl
10μg/ml
15μg/ml
20μg/ml
YPH277
wild type
kap123Δ
YPH278
wild type
kap123Δ
16°C
23°C
30°C
37°C
Temperature
YPH277
wild type
kap123Δ
YPH278
wild type
kap123Δ

viability for SAC mutants following treatment with nocodazole (Li and Murray, 1991; Straight and Murray, 1997). The ability of *kap123Δ* strains to remain viable following exposure to nocodazole suggests that these cells do not lose chromosomes as a result of microtubule disruption. Nonetheless, FACS analysis of *kap123Δ* cultures suggests a constitutive aneuploidy for this mutant (*Figure 3-2A*). In order to assess whether *kap123Δ* strains have a chromosome loss phenotype, we made *kap123Δ* mutant strains in the YPH277 and YPH278 strain backgrounds (Spencer et al., 1990; Kerscher et al., 2001). The application of these strains to assess chromosome loss is based on the fact that yeast strains carrying a mutation in the *ade2* gene develop red pigmentation. The strains YPH277 and YPH278 carry the ochre allele *ade2-101*, which, if expressed, causes yeast to be red. However, these strains also carry the ochre suppressor tRNA gene *SUP11* on a non-essential chromosome (Spencer et al., 1990). If chromosomes segregate normally, *SUP11* is maintained in all cells and the colonies remain white. However, if cells are prone to chromosome loss *SUP11* can be missegregated, resulting in red colonies or red and white (sectoring) colonies (Spencer et al., 1990). We plated serial dilutions of *kap123Δ* and wild type cells from the YPH277 and YPH278 parent strains onto complete medium or medium containing benomyl (*Figure 3-3B*). Yeast colonies from the YPH277 and YPH278 genetic backgrounds lacking Kap123p remained white on benomyl-containing medium at 30°C (*Figure 3-3B*; upper panel) and on complete medium at temperatures ranging from 16°C to 37°C (*Figure 3-3B*; lower panel). These results suggest microtubule disruption does not lead to a detectable chromosome loss in *kap123Δ* cells, supporting the premise that the SAC is functional in the absence of Kap123p.

3.2.3 The mitotic arrest elicited in response to microtubule disruption in *kap123Δ* cells resembles that of wild type cells

To further evaluate whether nocodazole-induced mitotic arrest in *kap123Δ* cells is comparable to that of wild type cells, we monitored the levels of the mitotic cyclin Clb2p in these cells following treatment with nocodazole. In wild type cells, levels of Clb2p begin to accumulate at the S/G2-phase transition and are stable until late anaphase (Baumer et al., 2000; Yeong et al., 2000; Peters, 2002; Wasch and Cross, 2002). The time-course of Clb2p accumulation and degradation can be used to evaluate progression through mitosis. In nocodazole-treated cells, activation of the SAC arrests cells in metaphase, stabilizing Clb2p for the duration of the arrest. To examine levels of Clb2p in *kap123Δ* mutant cells following a nocodazole-induced mitotic arrest, cultures were synchronized in G1-phase with α-factor and then released into medium with or without nocodazole. At various times after release from α-factor, total cell extracts were isolated and analyzed by Western blotting using antibodies specific for Clb2p. In the absence of nocodazole treatment, there does not appear to be a cell cycle arrest or delay in the *kap123Δ* mutant population (*Figure 3-4A*; upper panel). Based on Clb2p accumulation and degradation, *kap123Δ* and *mad2Δ* cells proceed through the cell cycle with timing similar to wild type cells (*Figure 3-4A*; upper panel). When cultures were treated with nocodazole we found that, as previously reported (Straight and Murray, 1997), the SAC

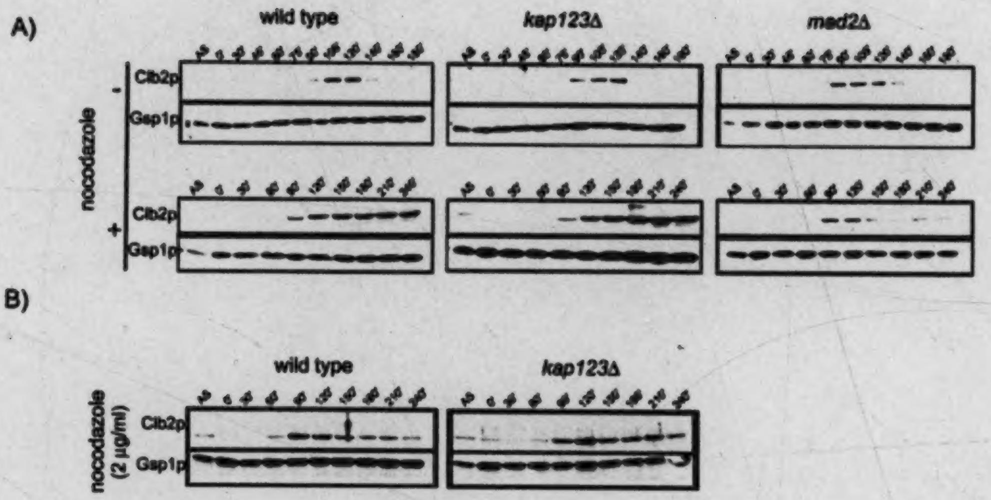


Figure 3-4. kap123Δ cells delay degradation of mitotic cyclins in response to nocodazole-induced microtubule disruption. **Panel A:** Accumulation and degradation of the mitotic cyclin Clb2p was used to examine progression through the cell cycle. Early log phase cultures of wild type (DF5a), *kap123Δ* (YAA3500), and *mad2Δ* (YMB1904) cells were arrested with α-factor and released into complete medium containing vehicle alone (DMSO) (-) or vehicle containing nocodazole (15 µg/ml) (+) at 30°C. Samples were taken at times indicated, total cell lysates were separated by SDS-PAGE and analyzed by Western blotting using antibodies directed against Clb2p and a load control Gsp1p. **Panel B:** *kap123Δ* cells delay degradation of mitotic cyclins in response to low concentrations of nocodazole. Wild type (DF5a) and *kap123Δ* (YAA3500) cells were sampled asynchronously, synchronized in G1 with α-factor and released into complete liquid medium containing 2 µg/ml nocodazole at 30°C. Samples were taken every 30 min for 4 h, separated by SDS-PAGE and analyzed by Western blotting, as above.

mutant *mad2Δ* cells do not maintain Clb2p in the presence of nocodazole (*Figure 3-4A*; lower panel). Rather, *mad2Δ* cells accumulate and degrade Clb2p with timing similar to that of *mad2Δ* cells released into medium lacking nocodazole (*Figure 3-4A*; upper panel). In contrast, *kap123Δ* cells, like wild type cells, maintain Clb2p for an extended period of time in response to nocodazole treatment (*Figure 3-4A*; lower panel).

It is possible that the SAC in *kap123Δ* mutant cells can respond to severe spindle disruption caused by the concentration of nocodazole used in the experiments above, but not to subtle microtubule defects. Low concentrations of nocodazole (< 4 μg/ml), which have relatively minor effects on the spindle itself, are postulated to affect microtubule-kinetochore interactions (Wang and Burke, 1995; Lee and Spencer, 2004). In order to assess the robustness of the SAC, we performed the Clb2p accumulation and degradation experiment as above, except that G1-phase arrested wild type and *kap123Δ* cells were released into medium containing a low concentration of nocodazole (2 μg/ml). As shown in *Figure 3-4B*, the accumulation and maintenance of Clb2p in response to low concentrations of nocodazole (2 μg/ml) is similar in wild type and *kap123Δ* cells (*Figure 3-4B*). From these data, we conclude that the absence of Kap123p does not affect the fidelity of the SAC under conditions of severe or subtle microtubule defects.

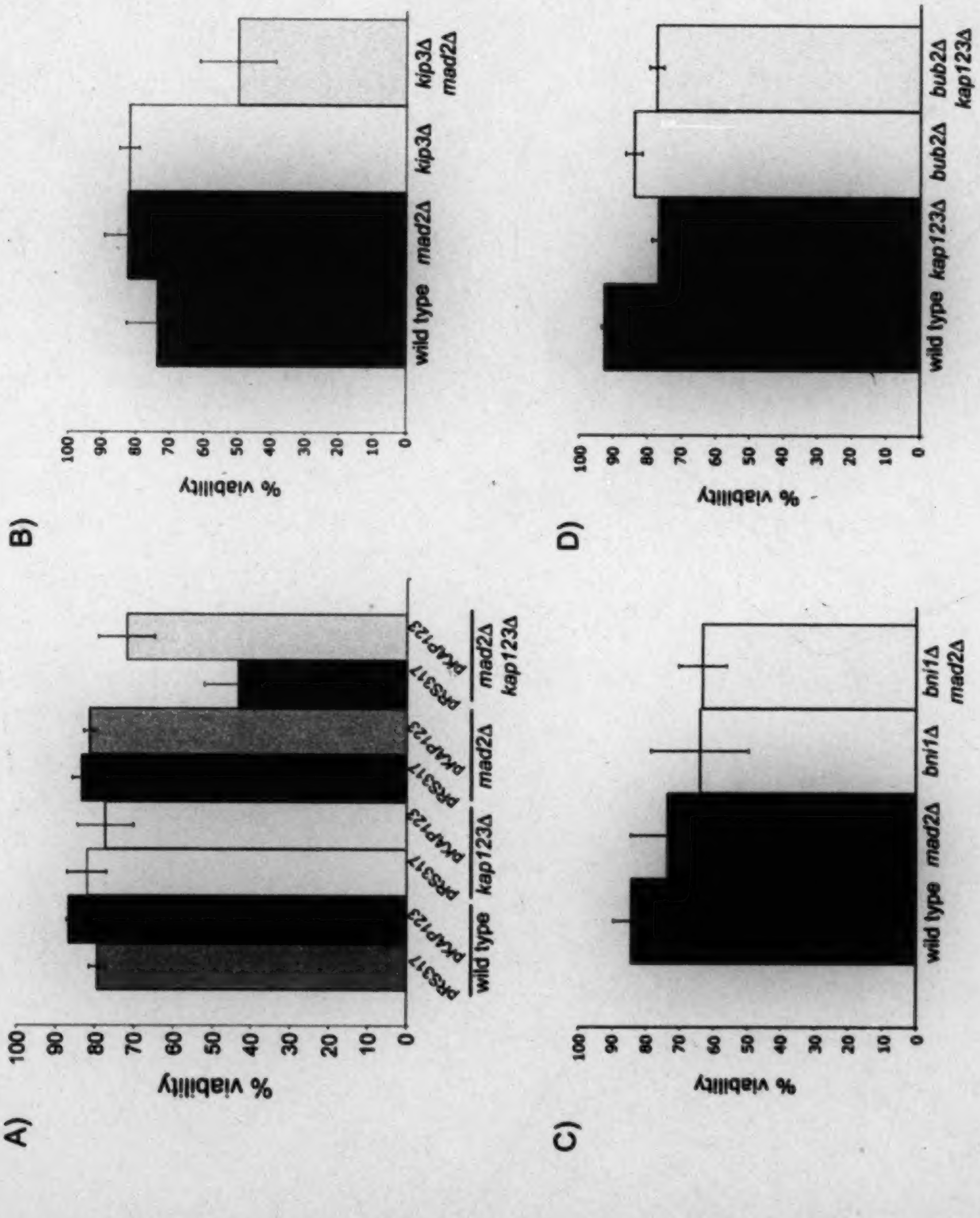
3.3 Kap123p appears to influence microtubule/spindle stability

3.3.1 *kap123Δ* cells lose viability in the absence of Mad2p

Since all of our data indicated that *kap123Δ* cells can arrest in response to microtubule damage, we considered it unlikely that defects in the SAC are the cause of the benomyl sensitivity observed for this mutant. Rather, we speculated that the benomyl sensitivity of *kap123Δ* strains might be due to impaired microtubule organization or stability. If microtubule abnormalities arise from the loss of Kap123p, we would predict that *kap123Δ* cells likely rely on a functional SAC to monitor microtubule/spindle function. To test this, we constructed a double mutant lacking both Kap123p and Mad2p and examined the viability of this strain.

For these studies, the viability of strains was assessed by removing cells from exponentially growing liquid cultures and placing individual cells in a grid pattern on complete medium using a dissecting microscope. Plates were then incubated at 30°C for 48 h. Percentage viability was calculated by dividing the number of colonies formed by the number of cells plated and multiplying by 100. As shown in *Figure 3-5A*, while cultures of the single mutant *kap123Δ* or *mad2Δ* cells display nearly wild type viability, the viability of the double mutant (*mad2Δ kap123Δ*) is only about 50% of wild type. Transforming *mad2Δ kap123Δ* cells with a plasmid-borne copy of *KAP123* restores viability (*Figure 3-5A*). These results imply that SAC function is required to maintain the viability of the *kap123Δ* strain. A similar viability loss has been identified in the absence of Mad2p for *gim* mutants with altered tubulin folding (Geissler et al., 1998),

Figure 3-5. kap123Δ cells lose viability in the absence of the SAC protein Mad2p. **Panel A:** The viability of wild type (DF5a), *kap123Δ* (YAA3500), *mad2Δ* (YMB1906), or *mad2Δ kap123Δ* (YAA3502) cells containing an empty vector (pRS317) or plasmid-borne *KAP123* (pRS317-*KAP123*) was assessed. Single cells were isolated from exponentially growing liquid cultures, manipulated into a grid pattern on YPD plates using a dissecting microscope and incubated at 30°C for 48 h. 50 cells were plated per experiment per strain; n=3. **Panel B:** The *kip3Δ* mutant exhibits decreased viability in the absence of Mad2p. Exponentially growing cultures of wild type (BY4741), *mad2Δ* (yjl030Δ), *kip3Δ* (ygl216Δ), or *kip3Δ mad2Δ* (YAA3520) strains were tested for viability as in panel A; n=5. **Panel C:** The viability of the *bni1Δ* mutant is unchanged in the absence of Mad2p. The viability of exponentially growing cultures of wild type (BY4741), *mad2Δ* (yjl030Δ), *bni1Δ* (ynl271Δ), or *bni1Δ mad2Δ* (YAA3521) cells was examined as above; n=5. **Panel D:** The viability of *kap123Δ* cells is unaffected by the loss of Bub2p. The viability of exponentially growing cultures of wild type (BY4741), *kap123Δ* (YAA3506), *bub2Δ* (ymr055Δ), or *bub2Δ kap123Δ* (YAA3525) cells was examined as above; n=3.


A)
% viability
100
90
80
70
60
50
40
30
20
10
0
pRS317
pKAP123
wild type
pRS317
pKAP123
kap123Δ
pRS317
pKAP123
mad2Δ
pRS317
pKAP123
mad2Δ
kap123Δ
B)
% viability
100
90
80
70
60
50
40
30
20
10
0
wild type
mad2Δ
kip3Δ
kip3Δ
mad2Δ
C)
% viability
100
90
80
70
60
50
40
30
20
10
0
wild type
mad2Δ
bni1Δ
bni1Δ
mad2Δ
D)
% viability
100
90
80
70
60
50
40
30
20
10
0
wild type
kap123Δ
bub2Δ
bub2Δ
kap123Δ

tubulin mutants (Hardwick et al., 1999; Tong et al., 2004), *cin8Δ* and *kar3Δ* microtubule motor mutants (Li and Murray, 1991; Geiser et al., 1997; Hardwick et al., 1999) mutants), and a *ctf13* kinetochore mutant (Wang and Burke, 1995). Therefore, there is a link between decreased viability in cells lacking Mad2p and requirement for the SAC resulting from microtubule/mitotic spindle defects. We speculated that analysis of other microtubule mutant strains may provide a gauge of mutations which require monitoring by Mad2p. For example, we detected viability loss comparable to that of the *mad2Δ kap123Δ* strain in the *kip3Δ mad2Δ* strain (*Figure 3-5B*) which is lacking the kinesin-type microtubule motor protein Kip3p. Kip3p was investigated because it reportedly functions in regulating spindle elongation, microtubule dynamics and spindle positioning (Cottingham and Hoyt, 1997; Miller et al., 1998; Cottingham et al., 1999). The formin Bni1p, which nucleates actin filaments, also mediates spindle positioning (Fujiwara et al., 1999; Lee et al., 1999; Miller et al., 1999). However, the viability of *bni1Δ* mutant cells is unaffected by the loss of Mad2p (*Figure 3-5C*). These data are consistent with a requirement for Mad2p in the presence of microtubule or mitotic spindle defects rather than spindle positioning defects. In contrast, the Bub2p-dependent pathway monitors spindle position (see section 1.6.2). We did not detect viability loss for the *kap123Δ* mutant in the absence of Bub2p (*Figure 3-5D*). This suggests the microtubule-related function of Kap123p is in a pathway monitored by the SAC rather than the spindle positioning checkpoint. The viability loss of *kap123Δ* cells in the absence of Mad2p is consistent with a role for Kap123p in mitotic spindle fidelity or another aspect of microtubule assembly, stability or dynamics.

Our data support the hypothesis that *kap123Δ* cells are benomyl sensitive due to compromised microtubule function. We speculated that if *kap123Δ* cells are benomyl sensitive because of a role for Kap123p in a microtubule-related process and *mad2Δ* cells are benomyl sensitive because of a role for Mad2p in the SAC, then the *mad2Δ kap123Δ* double mutant may exhibit a cumulative benomyl sensitivity more severe than that of the single mutant counterparts (Fraschini et al., 1999). To test this, we plated serial dilutions of wild type, *kap123Δ*, *mad2Δ*, and *mad2Δ kap123Δ* cells on medium containing benomyl. Consistent with Kap123p function in a microtubule-related pathway, double mutant *mad2Δ kap123Δ* cells display increased sensitivity to benomyl relative to either single mutant (*Figure 3-6A*). To address the specificity of this interaction, we tested whether the *kap123Δ* mutant also displays increased benomyl sensitivity in the absence of Swe1p or Bub2p, proteins involved in the morphogenesis checkpoint and the spindle positioning checkpoints, respectively. As shown in *Figure 3-6, B and C*, the loss of Swe1p or Bub2p has no effect on the benomyl sensitivity of *kap123Δ* cells. This suggests Kap123p acts in a pathway distinct from the SAC, but dependent on SAC function. Since the SAC monitors chromosome attachment to the mitotic spindle, this interaction is consistent with a role for Kap123p in augmenting mitotic spindle integrity via a microtubule-related process.

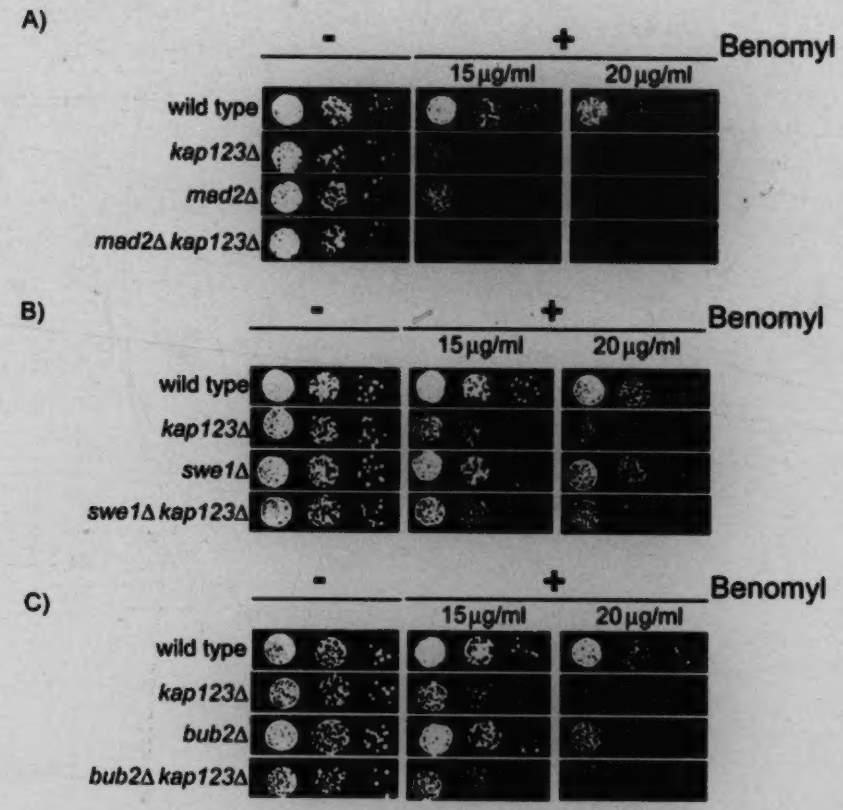

A)
-
+
Benomyl
15 µg/ml
20 µg/ml
wild type
kap123Δ
mad2Δ
mad2Δ kap123Δ
B)
-
+
Benomyl
15 µg/ml
20 µg/ml
wild type
kap123Δ
swe1Δ
swe1Δ kap123Δ
C)
-
+
Benomyl
15 µg/ml
20 µg/ml
wild type
kap123Δ
bub2Δ
bub2Δ kap123Δ

Figure 3-6. The benomyl sensitivity of kap123Δ cells is exacerbated in the absence of the SAC protein Mad2p. **Panel A:** *kap123Δ* cells lacking Mad2p show increased sensitivity to benomyl. Serial dilutions of wild type (DF5a), *kap123Δ* (YAA3500), *mad2Δ* (YMB1906), or *mad2Δ kap123Δ* (YAA3502) cells were spotted on YPD plates containing either vehicle alone (DMSO) (-) or vehicle plus increasing concentrations of benomyl (+). Plates were incubated at 30°C for 48 h. **Panels B and C:** The growth of *kap123Δ* cells lacking Swe1p or Bub2p is unchanged in the presence of microtubule-destabilizing drugs. Serial dilutions of wild type (BY4741), *kap123Δ* (YAA3506), *swe1Δ* (yjl187Δ), or *swe1Δ kap123Δ* (YAA3524) cells (panel B) or serial dilutions of wild type (BY4741), *kap123Δ* (YAA3506), *bub2Δ* (ymr055Δ), or *bub2Δ kap123Δ* (YAA3525) cells (panel C) were spotted on YPD (-) or YPD containing benomyl (+), as above.

3.3.2 Genetic Interactions of *KAP123*

The majority of *S. cerevisiae* genes are non-essential for growth, though impaired or absent cell growth can result from the loss of two or more genes whose products contribute to the same biological function (Tong et al., 2001; Tong et al., 2004). These genetic interactions are referred to as "synthetic sickness" or "synthetic lethality" and can be used to predict the involvement of interacting gene products in similar or related processes (Tong et al., 2001; Tong et al., 2004). Alternately, the loss of two or more genes whose products act antagonistically to one another may improve cell growth relative to the single mutant counterparts (Cottingham and Hoyt, 1997). Again, such interactions may be predictive of gene product function.

Consistent with a role for Kap123p in microtubule function or stability, *KAP123* reportedly shows a synthetic sick interaction with *GIM5* (Tong et al., 2004), which encodes a prefoldin sub-unit involved in tubulin and actin assembly (Geissler et al., 1998). Based on this observation, as well as our own data suggesting a novel microtubule-related role for Kap123p, we reasoned that identifying additional genetic interactions for *KAP123* could provide valuable insight into this process. To determine whether *KAP123* exhibits genetic interactions indicative of a role in microtubule or mitotic spindle function, a *kap123Δ* strain was crossed with several microtubule motor mutant strains, α-tubulin mutant strains, and spindle positioning mutant strains. We then tested growth of double mutants in the presence of benomyl and upon incubation at varying temperatures. The genetic interactions which we observed are summarized in *Table 3-1*. While we did not detect genetic interactions between *KAP123* and *KAR9*,

Table 3-1. KAP123 genetic interactions detected in this work.

KAP123 interacting	growth defect ?	Increased benomyl sensitivity relative to *kap123Δ* cells?	Decreased benomyl sensitivity relative to *kap123Δ* cells?
MAD2	yes – viability loss	Yes	no
KIP3	no	No	yes
BNI1	no	No	yes
TUB3	yes – slow growth in cold	No	no
TUB1	yes – slow growth in cold	No	no

DYN1, *MYO5* or *BEM2*, genes coding proteins involved in spindle positioning, we did observe genetic interactions consistent with a role for Kap123p in microtubule function.

We determined that the growth of *kap123Δ* cells on benomyl-containing plates can be restored to the level of wild type cells by knocking out *BNI1* or *KIP3*, genes which code for yeast formin and kinesin-related proteins, respectively (*Figure 3-7A*). We speculate that this interaction is indicative of antagonistic functions for Kap123p and Kip3p on microtubule dynamics. Kip3p is proposed to act as a microtubule-destabilizing factor (Cottingham and Hoyt, 1997; Miller et al., 1998; Cottingham et al., 1999). Reportedly, *kip3Δ* cells have longer than wild type microtubules and are more resistant to benomyl than wild type cells (Cottingham and Hoyt, 1997). Indeed, our data demonstrate that *kip3Δ* cells exhibit improved growth in benomyl-containing media, as do *bni1Δ* cells (*Figure 3-7A*). This suggests that cells lacking Bni1p may also have more stable microtubules than wild type cells. Consistent with this, Yeh et al. (2000) observed longer astral microtubules in *bni1Δ* cells than in wild type cells. This suggests that the improved

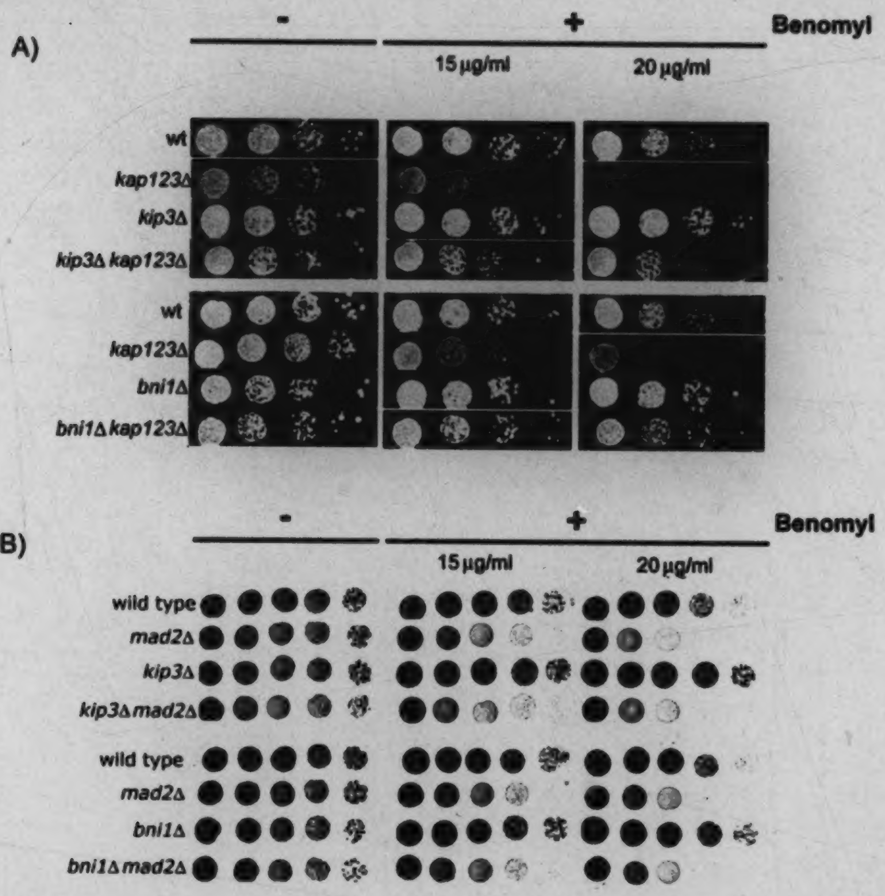

A)
-
+
Benomyl
15 µg/ml
20 µg/ml
wt
kap123Δ
kip3Δ
kip3Δ kap123Δ
wt
kap123Δ
bni1Δ
bni1Δ kap123Δ
B)
-
+
Benomyl
15 µg/ml
20 µg/ml
wild type
mad2Δ
kip3Δ
kip3Δ mad2Δ
wild type
mad2Δ
bni1Δ
bni1Δ mad2Δ

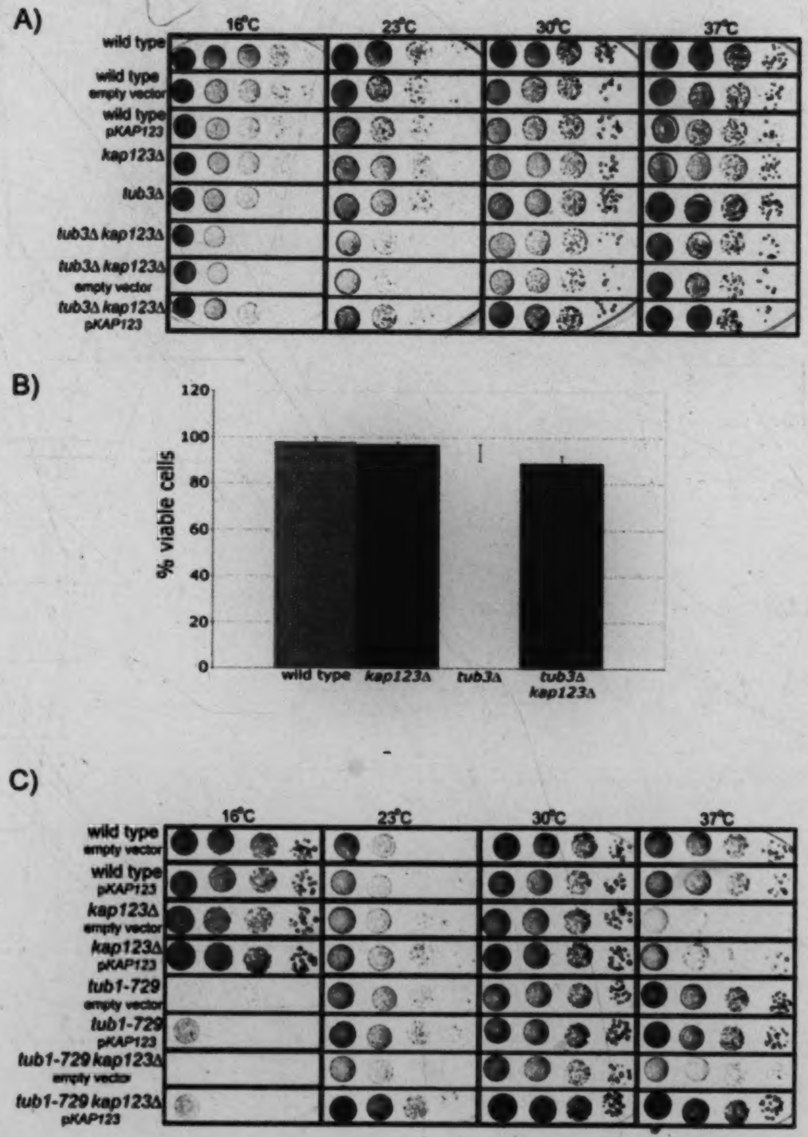
Figure 3-7. kap123Δ cells exhibit improved growth on benomyl-containing plates in the absence of Kip3p or Bni1p. **Panel A:** Serial dilutions of wild type (BY4741), *kap123Δ* (YAA3506), *bni1Δ* (ynl271Δ), or *bni1Δ kap123Δ* (YAA3523) cells (top panel) and of wild type (BY4741), *kap123Δ* (YAA3506), *kip3Δ* (ygl216Δ), or *kip3Δ kap123Δ* (YAA3522) cells (bottom panel) were spotted on YPD plates containing either vehicle alone (DMSO) (-) or vehicle plus increasing concentrations of benomyl (+). Plates were incubated at 30ºC for 48 h. **Panel B:** The loss of Bni1p or Kip3p does not improve the growth of all benomyl sensitive mutants grown in the presence of microtubule-destabilizing drugs. Serial dilutions of wild type (BY4741), *mad2Δ* (YAA3507), *bni1Δ* (ynl271Δ), or *bni1Δ mad2Δ* (YAA3521) cells (top panel) and of wild type (BY4741), *mad2Δ* (YAA3507), *kip3Δ* (ygl216Δ), or *kip3Δ mad2Δ* (YAA3520) cells (bottom panel) were spotted on YPD plates (-) or YPD containing benomyl (+), as above.

growth of *bni1Δ kap123Δ* cells on benomyl compared to the *kap123Δ* cells (*Figure 3-7A*) may be due to microtubule stabilization in *bni1Δ kap123Δ* cells relative to *kap123Δ* cells. If the *kip3Δ kap123Δ* mutant cells and *bni1Δ kap123Δ* mutant cells benefit from increased microtubule stability relative to the *kap123Δ* mutant, it is possible that microtubule stability is decreased in the absence of Kap123p. Kip3p (Cottingham and Hoyt, 1997; Miller et al., 1998) and Bni1p (Fujiwara et al., 1999; Lee et al., 1999; Miller et al., 1999) also influence spindle positioning in cells. However, we postulate that the improved growth of *kap123Δ* cells lacking Kip3p or Bni1p in the presence of benomyl is due to increased microtubule stability rather than altered spindle positioning (see discussion).

The improved growth of *kap123Δ* cells on benomyl-containing plates in the absence of *BNI1* or *KIP3* prompted us to investigate whether deleting these genes rescues the growth of other benomyl sensitive mutants. To address the specificity of the improved growth of *kap123Δ* cells lacking Bni1p or Kip3p in the presence of benomyl, we knocked out the *BNI1* or *KIP3* genes in conjunction with the SAC gene *MAD2*. As shown in *Figure 3-7B*, knocking out *BNI1* or *KIP3* does not rescue the growth of *mad2Δ* cells on benomyl-containing media. This suggests that improved growth in the presence of benomyl is not a general consequence of increasing microtubule stability. On the whole, these data implicate reduced microtubule stability as the cause of benomyl sensitivity for the *kap123Δ* mutant.

A number of genes encoding microtubule organization or stability factors exhibit genetic interactions with the tubulin genes (Hoyt et al., 1997; Geissler et al., 1998; Tong et al., 2004). Genetic interactions also exist amongst different tubulin genes (Richards et al., 2000). We hypothesized, based on our data, that *KAP123* might exhibit genetic interactions with genes coding tubulin monomers. Benomyl sensitivity is frequently associated with mutations affecting α-tubulin levels (Schatz et al., 1988; Hoyt et al., 1997; Geissler et al., 1998; Richards et al., 2000). Therefore, we tested for genetic interactions between *KAP123* and the α-tubulin genes *TUB1* and *TUB3* by constructing the double mutant strains *tub3Δ kap123Δ* and *tub1-729 kap123Δ* and testing each for growth defects. An intriguing genetic interaction was observed between *KAP123* and the non-essential α-tubulin gene *TUB3*. While *tub3Δ* cells exhibit a slight growth defect at 16°C, the double mutant *tub3Δ kap123Δ* cells display an exaggerated growth defect at this temperature (*Figure 3-8A*). The growth of *tub3Δ kap123Δ* cells is restored to that of the *tub3Δ* single mutant by transformation with a plasmid-borne copy of *KAP123* (*Figure 3-8A*). We next asked whether the growth defect of *tub3Δ kap123Δ* cells is a result of viability loss or of decreased growth rate. Following growth overnight at 16°C, individual cells from wild type, *tub3Δ*, *kap123Δ*, and *tub3Δ kap123Δ* cultures were placed in a grid pattern on complete medium using a dissecting microscope. We observed that the *tub3Δ kap123Δ* strain does not lose viability at 16°C (*Figure 3-8B*). Nearly 90% of *tub3Δ kap123Δ* cells were viable (*Figure 3-8B*). These data suggest that the microtubule defect(s) of *tub3Δ kap123Δ* cells may be more severe than those of *tub3Δ* cells, resulting in slow growth of this strain in the cold.

Figure 3-8. KAP123 shows a genetic interaction with the α–tubulin genes TUB1 and TUB3. **Panel A:** *tub3Δ* cells exhibit decreased growth at 16°C in the absence of Kap123p. Serial dilutions of wild type cells (YAA3529) and wild type cells (YAA3529) containing an empty vector (pRS317) or plasmid-borne *KAP123* (pRS317-*KAP123*), *kap123Δ* cells (YAA3530), *tub3Δ* cells (YAA3531), *tub3Δ kap123Δ* cells (YAA3527) and *tub3Δ kap123Δ* cells (YAA3527) containing an empty vector (pRS317) or containing plasmid-borne *KAP123* (pRS317-*KAP123*) were plated on YPD and incubated at temperatures between 16°C and 37°C. Plates were incubated for 48 h (23°C-37°C) or 5 d (16°C). **Panel B:** *tub3Δ kap123Δ* cells remain viable at 16°C. Wild type (BY4741), *kap123Δ* (YAA3506), *tub3Δ* (yml124Δ) and *tub3Δ kap123Δ* (YAA3527) cells were grown overnight in liquid medium at 16°C. Individual cells were isolated and manipulated into a grid pattern on YPD plates using a dissecting microscope. Plates were incubated at 30°C for 48 h. 50 cells were plated per strain per trial; n=3. **Panel C:** Plasmid-borne *KAP123* improves the growth of the *tub1-729* and *tub1-729 kap123Δ* mutant strains grown at 16°C and 23°C. Serial dilutions of wild type (DF5a), *kap123Δ* (YAA3500), *tub1-729* (DBY2411) and *tub1-729 kap123Δ* (YAA3528) cells containing an empty plasmid (pRS317) or plasmid-borne *KAP123* (pRS317-*KAP123*) were plated on YPD and incubated at temperatures between 16°C and 37°C. Plates were incubated for 48 h (23°C-37°C) or 5 d (16°C).


A)
16°C
23°C
30°C
37°C
wild type
wild type empty vector
wild type pKAP123
kap123Δ
tub3Δ
tub3Δ kap123Δ
tub3Δ kap123Δ empty vector
tub3Δ kap123Δ pKAP123
B)
% viable cells
120
100
80
60
40
20
0
wild type
kap123Δ
tub3Δ
tub3Δ kap123Δ
C)
16°C
23°C
30°C
37°C
wild type empty vector
wild type pKAP123
kap123Δ empty vector
kap123Δ pKAP123
tub1-729 empty vector
tub1-729 pKAP123
tub1-729 kap123Δ empty vector
tub1-729 kap123Δ pKAP123

We also tested for a genetic interaction between *KAP123* and the essential α-tubulin gene *TUB1* by knocking out *KAP123* in the cold sensitive *tub1-729* strain which exhibits decreased microtubule numbers at the non-permissive temperature (Schatz et al., 1988). To determine if the absence of Kap123p further compromises the growth of *tub1-729* cells, we plated serial dilutions of wild type, *kap123Δ*, *tub1-729*, and *tub1-729 kap123Δ* cells containing an empty vector or a plasmid-borne copy of *KAP123* on complete plate media. We incubated the plates at temperatures between 16°C and 37°C and assessed the growth of each strain. Consistent with reported data (Schatz et al., 1988), the *tub1-729* strain exhibits severe cold sensitivity at 16°C (*Figure 3-8C*). This growth defect is further exacerbated in the absence of Kap123p (*Figure 3-8C*). Interestingly, while a plasmid-borne copy of *KAP123* complements the growth of the *tub1-729 kap123Δ* strain, it also improves the growth of the *tub1-729* strain at 16°C and 23°C (*Figure 3-8C*). These data indicate that the absence of Kap123p decreases microtubule function, while extra copies of Kap123p improve the stability or organization of microtubules in this mutant. Taken together, these genetic interactions suggest a microtubule stabilizing role for Kap123p.

3.3.3 Physical interactions of Kap123p

In an effort to further characterize the basis of the observed functional interactions between Kap123p and proteins linked to microtubule stability, we have attempted to identify proteins that physically interact with Kap123p. To do this, cell lysates were made from yeast strains expressing *KAP123-PA*. Kap123-pA was then purified on IgG-Sepharose beads and the eluted fractions were analyzed by mass spectrometry. An untagged strain was used as a control for non-specific binding to the column. These experiments were performed in the laboratory of our collaborator Dr. John Aitchison at the Institute for Systems Biology (Seattle, WA). Among the peptides identified was one predicted to be derived from either Tub1p or Tub3p (due to sequence similarity, it was not possible to differentiate between the two α-tubulin proteins) and two predicted to be derived from the γ-tubulin protein Tub4p. We attempted to confirm the interactions between Kap123p and the tubulin proteins by Western blotting of affinity purified Kap123-pA fractions using antibodies specific for Tub1p or Tub4p. Unfortunately, we were unable to differentiate potential Tub1p or Tub4p bands from protein A degradation products present in the eluted fractions. The Kap123-pA affinity precipitation/mass spectrometry approach also identified peptides predicted to be derived from proteins related to microtubule function or organization. These proteins included several that have been previously suggested to interact physically or genetically with Tub1/Tub3p, Tub2p, Tub4p, or Gim5p.

3.3.4 Interaction Network

To gain insight into the molecular functions suggested by the genetic and physical interactions observed for Kap123p we incorporated protein interactions into one network. In order to integrate the genetic and physical interaction data that we, and others, have predicted or identified for Kap123p, we used Cytoscape interaction software (www.cytoscape.org) to create an interaction map. Physical interactions from our work are predicted based on Kap123-pA affinity precipitation/mass spectrometry data (see above). These interactions, as well as overlapping interactions reported by other groups and reported roles for interacting proteins are summarized in *Table 3-2*. Overlapping interactions between Kap123p and tubulin-related proteins described by us and others (*Table 3-2*) were manually entered into the Cytoscape program. In this interaction map (*Figure 3-9*) each connection (edge) indicates a genetic (pink) or physical (blue) interaction between individual proteins (nodes). Nodes were clustered manually. Nodes sharing multiple connections generally fall into distinct functional groups (Tong et al., 2001; Tong et al., 2004). As demonstrated by this interaction map, Kap123p shares numerous overlapping interactions not only with the tubulin proteins Tub1p, Tub3p, and Tub4p, but also with the actin and tubulin chaperonin component Gim5p (*Figure 3-9*). Overall, these overlapping physical and genetic interactions suggest potential functional relationships between Kap123p, tubulin, and tubulin-related proteins.

Table 3-2. Genetic and physical interactions for Kap123p overlapping with tubulin-related proteins.

	Reported Role(s)	Genetic	Physical	Reference
Acc1	-fatty acid biosynthesis (Roggenkamp et al., 1980)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Ahc1	-nucleosome disassembly (Eberharter et al., 1999)	*GIM5* (sick)	Kap123p	(Tong et al., 2004) (this work)
Bbc1	-actin cytoskeleton organization and biogenesis (Mochida et al., 2002; Tong et al., 2002)	*GIM5* (sick)	Kap123p	(Tong et al., 2004) (this work)
Bem2	-morphogenesis (Bender and Pringle, 1991)	*GIM5* (sick) *TUB2* (lethal)	Kap123p	(Tong et al., 2004) (Wang and Bretscher, 1995) (this work)
Bni1	-actin organization and nucleation (Evangelista et al., 2003)	*KAP123*		(this work)
Bub1	-spindle assembly checkpoint (Hoyt et al., 1991)	*GIM5* (lethal)	Kap123p	(Tong et al., 2004) (this work)
Caf40	-transcriptional regulation (Chen et al., 2001)	*GIM5* (sick)	Kap123p	(Tong et al., 2004) (this work)
Cap2	-actin capping (Kim et al., 2004)	*GIM5* (sick)	Kap123p	(Tong et al., 2004) (this work)

	Reported Role(s)	Genetic	Physical	Reference
Cct8	-cytoskeletal organization and biogenesis (Dunn et al., 2001)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Cdc48	-ubiquitin dependent protein catabolism (Rabinovich et al., 2002)		Tub4p Gim5p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002) (this work)
Chl1	-chromatid cohesion (Skibbens, 2004)	*GIM5* (sick) *MAD2* (sick)	Kap123p	(Tong et al., 2004) (Li and Murray, 1991; Tong et al., 2004) (this work)
Clu1	-translational initiation (Vornlocher et al., 1999), mitochondrion organization and biogenesis (Fields et al., 1998)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Elm1	-bud site selection, cytokinesis (Pruyne and Bretscher, 2000)	*GIM5* (sick) *TUB2* (lethal)	Kap123p	(Tong et al., 2004) (Parsons et al., 2004) (this work)
Gfa1	-cell wall biosynthesis (Lagorce et al., 2002)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Gim5	-tubulin and actin folding (Geissler et al., 1998)	*KAP123* (sick) *MAD2* (sick) *TUB3* (sick) *TUB1* (lethal) *TUB4* (lethal)		(Tong et al., 2004) (Tong et al., 2004) (Tong et al., 2004) (Vainberg et al., 1998) (Geissler et al., 1998)
Kap123	-nuclear transport (Rout et al., 1997; Schlenstedt et al., 1997)	*GIM5* (sick) *MAD2* (sick) *TUB3* (cold sensitive)	Tub3p Tub4p Kap123p	(Tong et al., 2004) (this work) (this work) (Gavin et al., 2002); (this work) (this work) (this work)
Kar2	-post-translational protein to membrane targeting (Young et al., 2001)		Tub3p Kap123p	(Hazbun et al., 2003) (this work)
Kip3	-microtubule based motor activity (Cottingham and Hoyt, 1997)	*KAP123* *MAD2*		(this work) (this work)
Mad2	-spindle assembly checkpoint (Li and Murray, 1991)	*KAP123* (sick) *TUB2* (sick/lethal) *GIM5* (sick)		(this work) (Hardwick et al., 1999; Lee and Spencer, 2004; Parsons et al., 2004) (Tong et al., 2004)
Nop1	-RNA processing, ribosomal sub-unit assembly (Venema and Tollervey, 1999; Dragon et al., 2002)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)

	Reported Role(s)	Genetic	Physical	Reference
Nop4	-RNA processing (Venema and Tollervey, 1999)		Tub3p Kap123p	(Gavin et al., 2002) (this work)
Pdc1	-ethanol fermentation (Eberhardt et al., 1999)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Pfk1	-glycolysis (Heinisch, 1986)		Tub1p Tub4p Gim5p Kap123p	(Schwock et al., 2004) (Gavin et al., 2002) (Gavin et al., 2002) (Gavin et al., 2002); (this work)
Prt1	-translational initiation (Phan et al., 1998)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Rpb2	-transcriptional regulation (Myer and Young, 1998)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Rpt6	-ubiquitin-dependent protein catabolism (Hochstrasser, 1996)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Rvs167	-bud site selection, response to osmotic stress (Pruyne and Bretscher, 2000)	*GIM5* (lethal)	Kap123p	(Tong et al., 2004) (this work)
Sam1	-methionine metabolism (Thomas and Surdin-Kerjan, 1991)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Sda1	-actin cytoskeleton organization and biogenesis (Buscemi et al., 2000)		Tub3p Kap123p	(Gavin et al., 2002) (this work)
Sec7	-ER to Golgi transport (Wolf et al., 1998)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Sec27	-ER to Golgi transport (Duden et al., 1994), retrograde transport (Letourneur et al., 1994)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Ski2	-translational regulation (Searfoss and Wickner, 2000)		Tub3p Kap123p	(Gavin et al., 2002) (this work)
Sla1	-actin organization and biogenesis (Pruyne and Bretscher, 2000)	*GIM5* (lethal)	Kap123p	(Tong et al., 2004) (this work)
Spf1	-ER function, calcium ion homeostasis (Cronin et al., 2002)	*GIM5* (sick)	Kap123p	(Tong et al., 2004) (this work)
Ssb1	-protein biosynthesis (Craig et al., 1993)	*GIM5* (sick)	Kap123p	(Siegers et al., 2003) (this work)
Ssb2	-protein biosynthesis (Craig et al., 1993)	*GIM5* (sick)	Kap123p	(Siegers et al., 2003) (this work)
Sum1	-chromatin silencing, transcriptional regulation (Chi and Shore, 1996)	*GIM5* (lethal)	Kap123p	(Tong et al., 2004) (this work)
Tif2	-translational regulation (Linder and Slonimski, 1989)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)

	Reported Role(s)	Genetic	Physical	Reference
Tub1	-cytoskeletal constituent (Nogales, 2001)	*TUB3* (sick/lethal) *TUB2* (lethal)	Tub2p Kap123p	(Richards et al., 2000) (Richards et al., 2000) (Kilmartin, 1981) (this work)
Tub3	-cytoskeletal constituent (Nogales, 2001)	*TUB1* (sick/lethal) *KAP123* (cold sensitive)	Kap123p	(Richards et al., 2000) (this work) (Gavin et al., 2002); (this work)
Tub4	-microtubule nucleation (Geissler et al., 1996; Marschall et al., 1996)	*GIM5* (lethal)	Kap123p Gim5p	(Geissler et al., 1998) (this work) (Gavin et al., 2002)
Uga1	-nitrogen utilization (Vissers et al., 1989)	*GIM5* (sick)	Kap123p	(Tong et al., 2004) (this work)
Utp22	-pre-rRNA processing (Peng et al., 2003)		Tub3p Kap123p	(Gavin et al., 2002) (this work)
Vid21	-chromatin modification (Nourani et al., 2003)	*MAD2* (lethal)	Kap123p	(Krogan et al., 2004; Tong et al., 2004) (this work)
Vma6	-vacuolar transport (Bauerle et al., 1993)	*GIM5* (sick)	Kap123p	(Tong et al., 2004) (this work)
Vps72	-chromatin remodelling (Krogan et al., 2003), protein vacuolar targeting (Bonangelino et al., 2002)	*GIM5* (lethal)	Kap123p	(Tong et al., 2004) (this work)
Ydj1	-ER associated protein catabolism (Huyer et al., 2004)	*TUB4* (lethal)	Tub3p Kap123p	(Oka et al., 1998) (Gavin et al., 2002) (Gavin et al., 2002); (this work)
Yef3	-translational elongation (McCarthy, 1998)		Tub3p Kap123p	(Gavin et al., 2002) (Gavin et al., 2002); (this work)
Ylr089C	-unknown	*GIM5* (lethal)	Kap123p	(Tong et al., 2004) (this work)
Ypl176C	-unknown	*GIM5* (sick)	Kap123p	(Tong et al., 2004) (this work)

3.3.5 Cells lacking Kap123p are prone to mitotic spindle defects

In an effort to visualize the effect of Kap123p on microtubule stability and dynamics, we used fluorescence microscopy to examine microtubules in wild type and *kap123Δ* cells expressing GFP-tagged α-tubulin. An integrating plasmid containing *GFP-TUB1* under

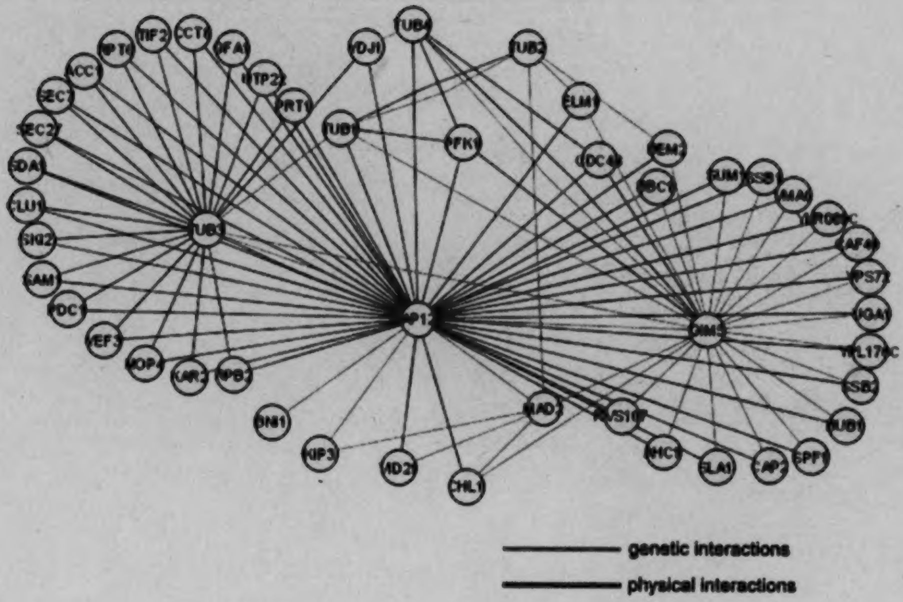
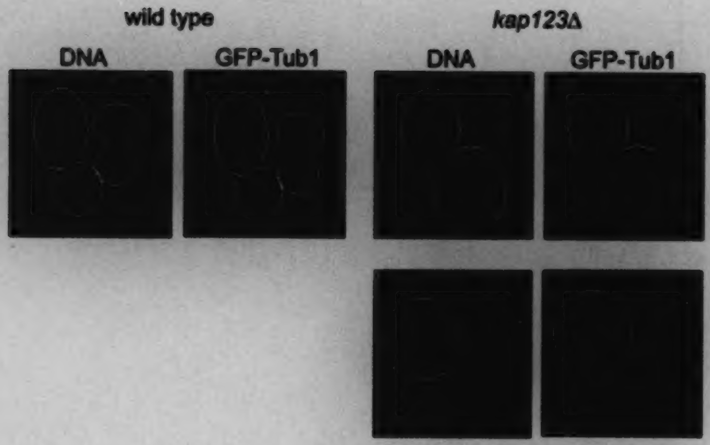

TUB4
YDJ1
TUB2
TIF2
CCT6
GFA1
RPT6
ACC1
HTP22
SEC7
PRT1
SEC27
TUB1
PFK1
ELM1
SDA1
CDC48
HEM2
CLU1
SBC1
RUM1
SSB1
TUB3
MAA
SKI2
YNR068C
SAF46
SAM1
VPS77
PDC1
KAP123
GIM5
HGA1
YEF3
YPL176C
MOP4
KAR2
RPB2
SSB2
MAD1
RVS167
TUB1
BNI1
NHC1
SLA1
CAP2
SPF1
KIP3
MD21
CHL1
genetic interactions
physical interactions

Figure 3-9. Interaction network for Kap123p. Data from this work were amalgamated with data reported by other groups (see *Table 3-2*) to visualize overlapping genetic and physical interactions of Kap123p, Tub1p, Tub3p, Tub4p, and Gim5p. Interactions were graphed using Cytoscape software (www.cytoscape.org). Each connection (edge) indicates a predicted genetic (pink) or physical (blue) interaction between individual proteins (nodes). Nodes were clustered manually. Physical interactions for Kap123p from our work are predicted based on mass spectrometric analysis of fractions eluted from affinity purified Kap123-pA generated by a yeast strain expressing *KAP123-PA*. These multiple interactions between Kap123p and microtubule-related proteins are consistent with a functional role for Kap123p in a microtubule-related process. These experiments were performed in the laboratory of our collaborator Dr. John Aitchison at the Institute for Systems Biology (Seattle, WA).

the control of the *MET3* promoter (Adames and Cooper, 2000) was transformed into wild type and *kap123Δ* cells. Cultures expressing GFP-Tub1p were synchronized in G1-phase using α-factor and released into complete media. Cells were taken from actively growing liquid cultures at various time points, co-stained for DNA and examined by fluorescence microscopy. Representative cells from a 120 min time point are shown (*Figure 3-10A*). Consistent with a function for Kap123p in microtubule organization, we observed an increase in the number of *kap123Δ* cells with aberrant or broken mitotic spindles (*Figure 3-10A*). A variety of mitotic spindle abnormalities were observed. One potential explanation for the diversity of these defects is that they may reflect changes to microtubule dynamics or to the forces being exerted on the microtubules of the mitotic spindle. This may lead to spindle collapse in some cells or to intact but abnormally shaped mitotic spindles in others.

An apparent consequence of these spindle defects is an increased frequency of bi- and multi-nucleated cells (*Figure 3-10A*). The existence of *kap123Δ* cells with more than one nucleus is consistent with FACS profiles showing greater than 2n DNA content for this mutant (*Figure 3-2A*). This suggests that *kap123Δ* cells may occasionally missegregate their entire genome or a large portion of it, leading to the excess DNA. Such nuclear missegregation likely occurs as a result of the microtubule instability which we propose for *kap123Δ* cells. A number of microtubule or mitotic spindle mutants also produce bi- and multi-nucleated cells. For example, nuclear missegregation can result from

A)



B)

	Single & Small Budded			Large Budded		
	●	●●	>2	●	●●	>2
wild type	98.7 +/- 0.5	1.3 +/- 0.5	0 +/- 0	98.7 +/- 0.5	1.3 +/- 0.5	0 +/- 0
kap123Δ	94.5 +/- 1.8	4.9 +/- 1.5	0.6 +/- 0.5	90.8 +/- 3.6	7.9 +/- 3.2	1.2 +/- 0.8

Figure 3-10. The kap123Δ strain displays a greater than wild type percentage of cells with aberrant mitotic spindle structures. **Panel A:** Microtubules were visualized by fluorescence microscopy of cells expressing GFP-Tub1p. Wild type (YAA3503) and *kap123Δ* (YAA3504) cells were transformed with an integrating plasmid containing *GFP-TUB1*. Expression of GFP-Tub1p was induced in CM-Met, cells were synchronized in G1 with α-factor and released into complete medium at 30°C. Samples were taken at various time points throughout the cell cycle and co-stained for DNA using Hoechst. Representative wild type and *kap123Δ* cells from samples taken 120 min following release from α-factor are shown. **Panel B:** Bi- and multi-nucleated cells result from aberrant mitotic spindle structures in *kap123Δ* cells. Samples were prepared as in panel A and single and small budded (left) or large budded (right) cells were scored for the presence of one, two, or more than two nuclei within one cell body. The percentage of each is indicated (90-1553 cells counted per experiment; n=7).

mutations affecting tubulin organization (Chen et al., 1994), microtubule nucleation (Chen et al., 1998; Knop and Schiebel, 1998), microtubule motor protein activity (Cottingham and Hoyt, 1997; Geiser et al., 1997), or spindle positioning (Knop and Schiebel, 1998). We determined the percentages of *kap123Δ* cells displaying this phenotype and found that, while the percentage of *kap123Δ* cells exhibiting binucleated cells is low (5-10%), it is significantly higher than the percentage of wild type cells with this phenotype (*Figure 3-10B*). Moreover, the prevalence of cells with bi- and multi-nucleated cells is comparable to that reported for mutants defective in tubulin chaperonin function (Chen et al., 1994), single mutants of specific microtubule motor or stability proteins (Cottingham and Hoyt, 1997; Geiser et al., 1997), or microtubule nucleation mutants (Chen et al., 1998). These results suggest that in the absence of Kap123p, microtubule structure or dynamics are altered in such a way that nuclear delivery is compromised.

CHAPTER FOUR: DISCUSSION

4.1 Cells lacking Kap123p have functional spindle assembly and spindle positioning checkpoints but are sensitive to microtubule-destabilizing drugs

We identified a previously unreported sensitivity for mutants lacking the β-kap Kap123p to the microtubule-destabilizing drug benomyl. This benomyl sensitivity appears to be specific to the loss of Kap123p as strains bearing mutations to or lacking the β-kaps Kap114p (*Figure 3-1B*), Kap104p, Msn5p, Los1p, Mtr10p, and Cse1p (R.J. Scott and R.W. Wozniak, unpublished) grow as wild type on benomyl-containing media. Therefore, we have characterized *kap123Δ* mutant cells in an attempt to determine the nature of Kap123p's involvement in a cellular pathway influencing growth in the presence of microtubule disruption.

Among mutations which lead to decreased growth in the presence of microtubule-destabilizing drugs are those which produce lesions in the SAC (Hoyt et al., 1991; Li and Murray, 1991; Yamamoto et al., 1996) or which affect tubulin organization, microtubule stability or microtubule dynamics (Huffaker et al., 1988; Schatz et al., 1988; Chen et al., 1994; Cottingham and Hoyt, 1997; Geiser et al., 1997; Geissler et al., 1998; Caron et al., 2001). We do not believe that the benomyl sensitivity of *kap123Δ* mutant cells is a result of a defective SAC in these cells. Unlike SAC mutant cells which exhibit rapid death in the presence of microtubule-destabilizing drugs (Straight and Murray, 1997), *kap123Δ* mutant cells remain viable in nocodazole (*Figure 3-3A*), suggesting that these cells have the ability to delay cell division in the absence of a functional mitotic spindle. Taken together, FACS analysis of DNA content (*Figure 3-2A*) and analysis of mitotic cyclin levels (*Figure 3-4, A and B*) of nocodazole-treated *kap123Δ* cells support the hypothesis that these cells are proficient to perform a mitotic arrest in response to microtubule disruption. Furthermore, the SAC in *kap123Δ* cells appears to be competent to perceive damage to the mitotic spindle and to elicit a checkpoint response in the presence of both severe and subtle microtubule/mitotic spindle defects (*Figure 3-4C*).

In our analysis of the checkpoint response to microtubule disruption in the *kap123Δ* mutant, we have focused on the SAC. However, the spindle positioning checkpoint (see section 1.6.2) also responds to microtubule defects which result in delayed spindle movement and nuclear delivery to the bud. In contrast to the SAC, the spindle positioning checkpoint arrests cells subsequent to the onset of anaphase spindle elongation. Nevertheless, the spindle assembly and spindle positioning checkpoints have some shared characteristics. Both influence degradation of mitotic cyclins by inhibiting APC components (Shah and Cleveland, 2000; Lew and Burke, 2003). Similar to SAC mutant cells, cells lacking the spindle positioning checkpoint components Bub2p or Bfa1p exhibit decreased growth in the presence of microtubule-destabilizing drugs (Hoyt et al., 1991; Li, 1999). In fact, Bub2p was initially identified as a component of the SAC (Hoyt et al., 1991). The observation that the *mad2Δ bub2Δ* double mutant strain is more sensitive to benomyl than either the *mad2Δ* or *bub2Δ* strains was a major criterion indicating that Bub2p is involved in a pathway distinct from that of Mad2p (Fraschini et al., 1999).

In our own analysis of double mutant strains grown on benomyl-containing plates, we did not see differences in growth between the *bub2Δ kap123Δ* and the *kap123Δ* strains (*Figure 3-6C*). This suggests that the growth of *kap123Δ* cells in the presence of microtubule-destabilizing drugs is not further compromised in the absence of Bub2p. Taken in isolation, these data might be interpreted as indicative of Kap123p's involvement in the Bub2p pathway. Based on other data, however, we do not believe this to be the case. For example, the Bub2p pathway acts upstream of mitotic cyclin Clb2p degradation and *bub2Δ* or *bfa1Δ* mutants fail to maintain levels of Clb2p in response to microtubule disruption (Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). Not surprisingly, *bub2Δ* cells, like SAC mutant cells, lose viability in nocodazole (Hoyt et al., 1991). If the reduced growth of *kap123Δ* cells in the presence of microtubule-destabilizing drugs results from defects to the Bub2p pathway, we would not expect to see the maintenance of Clb2p levels in *kap123Δ* cells (*Figure 3-4, A and B*) nor the survival of these cells following nocodazole treatment (*Figure 3-3A*). Therefore, we conclude that both the SAC and the spindle positioning checkpoint are functional in the absence of Kap123p.

4.2 Microtubule defects occur in the absence of Kap123p

A potential explanation for the decreased growth of *kap123Δ* cells on benomyl-containing plates in the absence of Mad2p, but not Bub2p (*Figure 3-6, A and C*), is that defects which exist in the absence of Kap123p require monitoring by the SAC but not the spindle positioning checkpoint. In vertebrate cells, the SAC appears to regulate the timing of anaphase during every cell cycle (Gorbsky et al., 1998). In contrast, yeast Mad1p and Mad2p are recruited to kinetochores to elicit a SAC arrest in response to specific mitotic spindle or kinetochore defects (Gillett et al., 2004). Subsequently, most of the SAC genes identified in yeast are non-essential in the absence of microtubule damage (Hoyt et al., 1991; Li and Murray, 1991). However, unlike wild type cells, the presence of Mad2p is required to maintain viability in *kap123Δ* cells (*Figure 3-5A*). This suggests the *kap123Δ* mutation leads to microtubule defects which are detected by the SAC. In contrast, the viability of *kap123Δ* cells was relatively unaffected in the absence of the spindle positioning checkpoint component Bub2p (*Figure 3-5, A and B*). Similarly, Wang and Burke (1995) demonstrated that Mad1p, Mad2p, Bub1p, and Bub3p, but not Bub2p, are required to maintain viability in a kinetochore mutant *ctf13* ts strain, which is defective for kinetochore-microtubule attachment at the non-permissive temperature (Wang and Burke, 1995). The compromised mitotic spindle function of this mutant apparently necessitates SAC activation. Genetic sick and lethal interactions have also been identified between the α- and β-tubulin genes *TUB3* and *TUB2* and the SAC genes *MAD1*, *MAD2*, and *MAD3* (Hardwick et al., 1999; Lee and Spencer, 2004; Tong et al., 2004). To date, no genetic interactions have been reported between these tubulin genes and *BUB2*. In contrast, a strain bearing a temperature sensitive mutation in *spc72* (see section 1.5.2), which results in mislocalization of the Tub4p microtubule-nucleating complex from the outer plaque of the SPB at the non-permissive temperature, loses viability in the absence of Bub2p but not Mad2p (Pereira et al., 2000). By affecting astral microtubule nucleation, the *spc72* mutation leads to spindle positioning defects which requires monitoring by the spindle positioning checkpoint (Pereira et al., 2000). Based on these findings, the requirement for Mad2p but not Bub2p in *kap123Δ* cells suggests that cells lacking Kap123p have a defect in general microtubule structure and/or fidelity of the mitotic spindle.

As discussed, the requirement for Mad2p in *kap123Δ* cells suggests the SAC monitors microtubule defects inherent in *kap123Δ* cells. However, we predict that cells with defective microtubule structures would exhibit a SAC-mediated metaphase arrest. Based on monitoring of the timing of the accumulation and degradation of the mitotic cyclin Clb2p in synchronized *kap123Δ* cells (*Figure 3-4A*), this mutant does not have a detectable cell cycle delay/arrest. This is not completely unexpected, however, as a similar phenomenon has been described for cells lacking proteins of the GimC prefoldin complex (Geissler et al., 1998). In the absence of a fully functional GimC-complex, cells are defective in tubulin biogenesis (Geissler et al., 1998). Not surprisingly, genes coding proteins of this complex exhibit synthetic sick or lethal interactions with the SAC genes *MAD1*, *MAD2*, and *MAD3* (Tong et al., 2004) similar to viability loss we observed for the *mad2Δ kap123Δ* mutant (*Figure 3-5A*). Nevertheless, *gim* mutant strains do not arrest at a defined stage of the cell cycle even though these mutants are competent to elicit a SAC response to nocodazole-induced microtubule disruption (Geissler et al., 1998). This similar phenotype may indicate a role for Kap123p in a GimC-related tubulin folding pathway (see section 4.2.2). On the other hand, the absence of Kap123p or GimC-complex components may simply result in microtubule defects of comparable severity. In either case, we speculate that if the SAC is required to monitor microtubules in *kap123Δ* cells, such activity is extremely transient. It is possible that the subtlety of the microtubule defects in *kap123Δ* cells necessitates only a short delay in the cell cycle to repair the majority of the microtubule defects.

4.2.1 Kap123p appears to influence microtubule stability

Our analysis of *kap123Δ* mutant cells has led to the hypothesis that Kap123p influences microtubule stability. This idea is supported by functional interactions between Kap123p and proteins with recognized roles in microtubule dynamics. We believe the genetic interactions observed for the *kip3Δ kap123Δ* and *bni1Δ kap123Δ* double mutants (*Figure 3-7A*) reflect antagonistic functions for Kap123p relative to Kip3p and Bni1p. Kip3p is a kinesin-related motor protein proposed to possess microtubule-destabilizing activity (Cottingham and Hoyt, 1997; Miller et al., 1998). In the absence of Kip3p, microtubules are longer than wild type and cells display enhanced growth in benomyl-containing medium (Cottingham and Hoyt, 1997). In agreement with this, we observed benomyl resistant growth for the *kip3Δ* mutant relative to wild type cells (*Figure 3-7A*). As we were able to restore the growth of *kap123Δ* cells to that of wild type cells in medium containing benomyl by knocking out *KIP3* (*Figure 3-7A*), we speculate that Kap123p and Kip3p may act in pathways functioning antagonistically to one another with respect to microtubule stability. This suggests that Kap123p directly or indirectly stabilizes microtubules.

While the improved growth of the *kip3Δ kap123Δ* double mutant strain, relative to the *kap123Δ* strain, in the presence of microtubule-destabilizing drugs may reflect microtubule stabilization in the absence of Kip3p, interpreting the improved growth of the *bni1Δ kap123Δ* mutant on benomyl-containing plates is not as straightforward. Bni1p is a formin which nucleates the actin cytoskeleton (reviewed in Evangelista et al., 2003). It is plausible that, like the *kip3Δ kap123Δ* strain, the improved growth of the *bni1Δ kap123Δ* strain on benomyl-containing plates is due to the presence of more stable microtubules in cells lacking Bni1p. Like *kip3Δ* mutant cells, *bni1Δ* mutant cells exhibit improved growth in the presence of benomyl compared to wild type cells (*Figure 3-7A*). This may indicate increased microtubule stability in cells lacking Bni1p. There is some evidence that *bni1Δ* mutants have longer astral microtubules than wild type cells (Yeh et al., 2000) and that these longer microtubules display exaggerated interactions with the bud neck (Segal and Bloom, 2001). However, no mechanistic explanation for these longer microtubules has been suggested other than the assertion they may be an indirect consequence of altered spindle positioning in the absence of Bni1p (Yeh et al., 2000).

Interestingly, the spindle positioning responsibilities of Bni1p occur within a pathway involving Kip3p. Kip3p and Bni1p are proposed to orient the mitotic spindle by arbitrating interactions between microtubules and the cell cortex (Lee et al., 1999). Therefore an alternate explanation for the alleviation of benomyl sensitivity exhibited by *kap123Δ* cells in the absence of Kip3p or Bni1p (*Figure 3-7A*) is that Kap123p function influences mitotic spindle positioning. However, based on our data, we do not believe this is the case. We did not detect genetic interactions between Kap123p and several other spindle positioning proteins. As well, unlike the spindle positioning mutant *spc72-10*, which requires the spindle positioning checkpoint component Bub2p to survive

(Pereira et al., 2000), we demonstrate that *kap123Δ* cells do not lose viability in the absence of Bub2p (*Figure 3-5B*).

Further supporting our proposal that Kap123p influences microtubule stability are the genetic interactions observed between *KAP123* and the α-tubulin genes *TUB1* and *TUB3* (*Figure 3-8*). A number of tubulin mutants display a characteristic cold sensitivity (Huffaker et al., 1988; Schatz et al., 1988; Hoyt et al., 1997). Because the absence of Kap123p exacerbates the growth defect of *tub3Δ* cells at 16°C without decreasing the viability of these cells (*Figure 3-8, A and B*), we believe microtubule structures in *tub3Δ* cells are further compromised in the absence of Kap123p. This also supports our conclusion that the SAC is functional in the absence of Kap123p, since abrogation of SAC function in combination with a *tub3Δ* mutation results in viability loss (Lee and Spencer, 2004; Tong et al., 2004). Though we have focused on the genetic interaction detected between *KAP123* and *TUB3*, we also observed a visible decrease in the growth of *tub1-729 kap123Δ* cells at 16°C relative to *tub1-729* cells (*Figure 3-8C*). This synthetic growth defect, as well as the growth of the *tub1-729* single mutant strain, can be rescued by the introduction of a plasmid-borne copy of *KAP123* (*Figure 3-8C*). These interactions, while subtle, suggest that Kap123p function may increase the ability of microtubules to withstand perturbation. Numerous cold sensitive *tub1* mutant alleles have been constructed and classified based on the microtubule defects observed in strains expressing these alleles (Schatz et al., 1988). It may be useful in the future to screen for other synthetic genetic interactions that might exist between *kap123Δ* and *tub1* mutant alleles. It is possible that these interactions may fall into particular functional groups and may assist in more specifically identifying the pathway(s) through which Kap123p impinges on microtubule function.

Bode et al. (2003) have suggested, based on *in vitro* analysis of purified tubulin, that Tub3p has a role in microtubule dynamics. When the authors purified tubulin from *tub1* mutant yeast strains overexpressing *TUB3* and reassembled microtubules *in vitro*, the resulting microtubules displayed less frequent shrinkage than microtubules assembled from tubulin derived from wild type cells (Bode et al., 2003). Conversely, tubulin purified from *tub3Δ* cells, in which Tub1p was the only source of α-tubulin, produced microtubules which underwent increased shrinkage and catastrophe (Bode et al., 2003). If *in vivo* microtubules behave analogously to those described *in vitro*, microtubules in the *tub3Δ* strain are predicted to undergo increased disassembly and more frequent conversion from growth to shrinkage phases. A more comprehensive understanding of *KAP123* genetic and physical interactions with the α-tubulin proteins may be useful for directing future studies of Kap123p. For example, it growth defects are more severe for the *tub3Δ* mutant than for various *tub1* mutants in the absence of Kap123p, it would suggest that the loss of Kap123p hastens microtubule disassembly already occurring in the *tub3Δ* mutant.

4.2.2 Potential causes of decreased microtubule/mitotic spindle stability in *kap123Δ* cells

There are a number of pathways through which Kap123p may influence microtubule organization or the production of a fully functional mitotic spindle. Here we discuss our findings in the context of the potential involvement of Kap123p in pathways influencing tubulin biogenesis, microtubule nucleation/polymerization, or microtubule motor protein activity.

4.2.2.1 Potential role for Kap123p in Tubulin Biogenesis

The *GIM* ("genes involved in microtubule biogenesis") gene products were identified as members of a pathway leading to the correct folding of actin and tubulin monomers (Geissler et al., 1998; Siegers et al., 2003). Intriguingly, Tong and co-workers (2004) identified a synthetic sick interaction between null mutants of *KAP123* and *GIM5* using synthetic genetic array analysis. *GIM5* is described as a "hub gene" because it genetically interacts with genes coding for proteins involved in microtubule dynamics, actin assembly, the dynein/dynactin pathway, the SAC, the DNA replication checkpoint, sister chromatid cohesion, and the chitin synthase III pathway, (Tong et al., 2004). The synthetic growth defect of the *kap123Δ gim5Δ* strain is suggestive of a functional relationship between Kap123p and Gim5p. We did not detect peptides predicted to be derived from Gim5p in Kap123-pA affinity precipitation experiments (*Figure 3-9*; *Table 3-2*). However, a number of proteins which interact genetically with Gim5p were identified (*Figure 3-9*). This indicates Kap123p may act in a pathway parallel to Gim5p, contributing to a shared function. In other words, Kap123p may physically interact and function in concert with proteins involved in a Gim5p-overlapping process. The loss of genes coding any of these proteins may lead to growth defects in combination with a *gim5Δ* mutation due to concurrent defects in two functionally overlapping pathways. Furthermore, the *kap123Δ* mutant strain shares some notable characteristics with *gim* mutant strains. Both depend on the SAC for viability but neither exhibits the cell cycle arrest anticipated by this genetic interaction (see section 4.2). Additionally, *kap123Δ* cells have a propensity to become larger than wild type cells (Schlenstedt et al., 1997), a phenotype associated with actin and actin biogenesis mutants. Similarly, due to the role of the GimC-complex in actin organization, the *gim* mutant cells also tend to be larger than wild type cells (Geissler et al., 1998).

4.2.2.2 Potential role for Kap123p in Microtubule Nucleation or Mitotic Spindle Polymerization

It is also plausible that Kap123p shares an overlapping function with Gim5p in a pathway distinct from the tubulin biogenesis. In yeast, genes coding the GimC-complex components were identified using a screen for mutations which are synthetically lethal in combination with a γ-tubulin (*tub4*) temperature sensitive allele (Geissler et al., 1998). Furthermore, Geissler and co-workers (1998) demonstrated by co-immunoprecipitation that Gim1p/Yke2p, Gim2p/Pac10p, Gim3p, Gim5p interact with overproduced Tub4p. This suggests that the GimC- and Tub4p-complexes may participate in a common function. It is unclear whether this function is related to the chaperone function of the GimC-complex or the microtubule nucleation function of the Tub4p-complex. The identification of peptides predicted to be derived from Tub4p by mass spectrometric analysis of fractions eluted from a Kap123-pA affinity precipitation experiments (*Table 3-2*; *Figure 3-9*) may implicate Kap123p in a microtubule process related to Tub4p. Tub4p localizes to the SPB where it functions in the nucleation of astral and nuclear microtubules (Geissler et al., 1996; Marschall et al., 1996) (see section 1.5.3). It is possible that Kap123p influences this function through transport of Tub4p to the SPB or through an alternate, non-transport pathway. However, our data argue against this explanation. Tub4p is reportedly transported to the nucleus in a complex with Spc97p and Spc98p, directed by an NLS in Spc98p (Pereira et al., 1998). We did not detect peptides predicted to be derived from Spc97p or Spc98p by mass spectrometric analysis of affinity purified Kap123-pA interacting fractions. Additionally, the phenotype(s) associated with *tub4* mutations are dissimilar to those observed for *kap123Δ* mutants. For example, in contrast to *kap123Δ* mutants, a number of *tub4* mutants are resistant to or exhibit wild type growth in the presence of benomyl (Spang et al., 1996; Vogel and Snyder, 2000). For example, cells expressing a deletion mutant of Tub4p lacking the conserved C-terminal region display temperature sensitive growth which is suppressed in the presence of the microtubule-destabilizing drug benomyl (Vogel and Snyder, 2000).

The Tub4p-complex functions in microtubule nucleation from the SPB. Similarly, γ-tubulin nucleates microtubules from the microtubule organizing centre (MTOC) of higher eukaryotes (see section 1.5.3). However, additional systems, such as chromatin-induced microtubule polymerization (Karsenti et al., 1984), also contribute to microtubule organization and production of mitotic spindles in higher eukaryotes (Carazo-Salas et al., 2001; Gruss et al., 2001; Nachury et al., 2001). It was recently demonstrated that kaps and Ran have regulatory roles in this chromatin-induced mitotic spindle assembly (see section 1.4.3) (Carazo-Salas et al., 2001; Gruss et al., 2001; Nachury et al., 2001). Chromatin-bound RCC1 (Ohtsubo et al., 1989), the Ran-GEF (Bischoff and Ponstingl, 1991a; Bischoff and Ponstingl, 1991b), increases the concentration of Ran-GTP in the vicinity of chromatin (Kalab et al., 2002). Ran-GTP then stimulates the release of proteins promoting microtubule polymerization from Kap-α and Kap-β1 (Carazo-Salas et al., 2001; Gruss et al., 2001; Nachury et al., 2001). To date, no such mechanism has been identified in *S. cerevisiae*. However, the Mad2p-dependent cell cycle arrest observed for *ntf2-2* mutants (Quimby et al., 2000), in which Ran is depleted from the nucleus (Quimby et al., 2005), alludes to spindle damage in these cells. Indeed, a version of the kap-Ran spindle regulatory system (Carazo-Salas et al., 2001; Gruss et al., 2001; Nachury et al., 2001) may exist in yeast. If so, kaps may function in cell cycle regulated targeting of microtubule polymerizing proteins to chromosomes to assist in mitotic spindle assembly. In this case, rather than releasing cargo immediately upon entering the nucleoplasm, kaps may sequester microtubule polymerizing proteins, only releasing them near chromosomes during mitotic spindle assembly. In the presence of an intact NE during mitosis, as in yeast, this implies an intra-nuclear targeting by kaps between the nuclear face of the NPC and chromosomes (see section 4.4.1). If kaps and Ran do influence chromatin-induced microtubule assembly in yeast, the microtubule defects and aberrant mitotic spindle structures which we have observed in *kap123Δ* cells may be due to defective Kap123p-mediated targeting of spindle polymerization factors.

4.2.2.3 Potential role for Kap123p in Targeting Microtubule Motors

In *Xenopus*, microtubule motors are among the proteins targeted to chromatin by the Ran-mediated spindle assembly system. For example, TPX2, an aster promoting protein released from Kap α (importin α) in *Xenopus*, is a targeting factor for the kinesin-related microtubule motor protein Xklp2 (Gruss et al., 2001). Microtubule motors influence microtubule structure, stability, dynamics as well as mitotic spindle assembly and elongation (Cottingham and Hoyt, 1997; Saunders et al., 1997; Straight et al., 1998; Cottingham et al., 1999; Yeh et al., 2000; Requena et al., 2001). Therefore, our hypothesized role for Kap123p in influencing microtubule stability may indicate that Kap123p functions in a microtubule motor protein regulatory pathway. Kap123p may mediate the nuclear targeting of one or more microtubule motor proteins (see section 4.4.1) or influence motor protein activity by an alternate mechanism. This is consistent with the genetic interaction which we have detected between *KAP123* and the microtubule motor protein *KIP3* (*Figure 3-7A*; see above). Similar to *kap123Δ* mutants (*Figure 3-5A*), several microtubule motor protein mutants depend on SAC functionality for survival (Geiser et al., 1997; Hardwick et al., 1999). In particular, the *kap123Δ* mutant strain shares several characteristics with the *kip2Δ* microtubule motor mutant strain. Both are sensitive to the microtubule-destabilizing drug benomyl, appear to have less stable microtubules, and exhibit an increased production of binucleate cells (Cottingham and Hoyt, 1997). Similar to *kap123Δ* cells, the growth of *kip2Δ* cells in the presence of benomyl can be rescued by knocking out *KIP3* (Cottingham and Hoyt, 1997). There are differences in the phenotypes of *kap123Δ* and *kip2Δ* cells (Cottingham and

Hoyt, 1997). We determined that 5-10% of *kap123Δ* cells contain two nuclei within one cell body. While the percentage of binucleate *kip2Δ* cells is low at ambient temperature, nearly 30% of *kip2Δ* cells are binucleate at 12°C (Cottingham and Hoyt, 1997). We speculate that the relatively low percentage of *kap123Δ* cells with detectable microtubule defects or binucleate cells (*Figure 3-10, A and B*) may be due to functional overlap in Kap123p-mediated processes influencing microtubule dynamics.

4.3 Functional overlap exists in pathways governing both nuclear transport and microtubule organization

Because microtubules constitute the mitotic spindle which physically segregates chromosomes, microtubule function is imperative for the completion of accurate cell division. This raises the question of why a non-essential protein such as Kap123p (Rout et al., 1997; Schlenstedt et al., 1997) is involved in this process. We do not consider the non-essentiality of Kap123p contradictory to our hypothesized role for Kap123p in microtubule function. Rather, we speculate that other proteins may be able to partially substitute for Kap123p in microtubule-related pathways. Such functional overlap exists both for nucleocytoplasmic transport pathways (Rout et al., 1997; Mosammaparast et al., 2002; Leslie et al., 2004) and for pathways leading to microtubule and mitotic spindle function (Geiser et al., 1997; Geissler et al., 1998; Straight et al., 1998; Cottingham et al., 1999). The microtubule-related function of Kap123p likely shares functional overlap with other proteins to insulate cells from severe microtubule defects in the absence of Kap123p. This is consistent with the subtle microtubule defects which we observe for *kap123Δ* cells. Similarly, ribosomal biogenesis is essential for cell survival, yet the import of ribosomal proteins to the nucleus for maturation is largely mediated by Kap123p (Rout et al., 1997; Schlenstedt et al., 1997). In this case, Kap121p partially substitutes for Kap123p nuclear transport (Rout et al., 1997). Whether the microtubule-related function of Kap123p is transport related or distinct from transport (see section 4.4), other protein(s) likely substitute for Kap123p function. Similar to nuclear transport pathways, remarkable functional overlap exists for proteins involved in microtubule and mitotic spindle regulation. For example, the system by which tubulin becomes folded and functional is mediated by both the GimC complex and the CCT complex. All genes coding for GimC-complex components are non-essential in *S. cerevisiae*, though subtle defects occur as a result of lesions to this pathway (Geissler et al., 1998). Similarly, Cottingham and co-workers (1999) demonstrated that *S. cerevisiae* cells are capable of survival in the absence of five of the seven microtubule motors identified in that organism. Though cells exhibit defects in progression through mitosis and spindle positioning, cells can grow with only Cin8p and either Kar3p or Kip3p microtubule motor proteins present (Cottingham et al., 1999).

4.4 Kap123p may Influence Microtubule Function Through a Transport or Non-Transport Pathway

We have speculated, to this point, about the nature of the microtubule defects in *kap123Δ* cells without specifically addressing the fundamental question of whether these defects result from loss of Kap123p-mediated transport or of a distinct process involving Kap123p. We speculate that Kap123p may influence microtubule stability by facilitating the nuclear or intra-nuclear transport of specific cargo(es) or, alternatively, through a more direct effect on microtubule structure.

Based on the characterized role of Kap123p as a nuclear transport factor (Rout et al., 1997; Schlenstedt et al., 1997), it is possible that Kap123p influences microtubule stability by nuclear delivery of cargo(es) important for microtubule function. If the defect of *kap123Δ* cells on benomyl-containing plates is related to transport, the pathway in question is not likely one for which Kap121p can substitute (*Figure 3-1C*). Kap123p transport pathways may overlap with those of kaps other than Kap121p (Sydorskyy et al., 2003). However, even in the presence of overlapping transport pathways, the absence of Kap123p may result in decreased accumulation of microtubule regulatory cargo(es) in the nucleus, leading to the observed microtubule defects. The defects detected in *kap123Δ* cells suggest that these cargo(es) may include regulatory microtubule motor proteins or other microtubule associated proteins. For example, the microtubule motor proteins Kip1-3p, Kar3p, and Cin8p all localize, at least partially, to SPBs and the mitotic spindle (Huh et al., 2003) suggesting that they require targeting to the nucleus. As discussed (section 4.2.2), the *kap123Δ* mutant has a phenotype similar to that of the *kip2Δ* mutant. Theoretically, this similarity may be due to decreased nuclear accumulation of Kip2p in the absence of Kap123p.

Kap123p-mediated delivery of microtubule regulatory protein(s) may occur through a traditional nuclear transport pathway or alternately, through a novel, intra-nuclear transport pathway, facilitating the delivery of microtubule/mitotic spindle organizing proteins, not only to the nucleoplasm, but to the site of mitotic spindle assembly and/or kinetochore attachment. As described (section 4.2.2.2), it is possible that yeast cells employ a kap-Ran system for mitotic spindle assembly analogous to that of *Xenopus*. Because yeast cells undergo closed mitosis, with no NE breakdown, such a system may involve cell-cycle regulated, intra-nuclear delivery of cargo to the site of mitotic spindle formation. Rather than releasing cargo immediately upon entering the nucleoplasm, importing kaps, such as Kap123p, may sequester microtubule polymerizing factors until encountering Ran-GTP in the vicinity of chromosome-bound Ran-GEF, Prp20p (Lee et al., 1993).

Alternatively, the microtubule-related function of Kap123p may be independent of both nuclear transport and of Ran. Kap123p exhibits a broad distribution within the cell, localizing to the cytoplasm, nucleus, and NPC (Rout et al., 1997; Schlenstedt et al., 1997). Functional interactions between Kap123p and Gim5p (Tong et al., 2004), which is a member of an exclusively cytoplasmic complex (Geissler et al., 1998; Siegers et al., 1999; Siegers et al., 2003), suggest Kap123p may have cytoplasmic functions beyond the recognition of nuclear import cargo. If so, these may include functions which influence microtubule organization.

4.5 Overview

We have uncovered a novel function for the *S. cerevisiae* kap Kap123p in influencing the stability of microtubules. The loss of Kap123p results in a decreased ability of cells to grow in the presence of microtubule-destabilizing drugs. We do not believe this drug sensitivity is due to abrogation of either the SAC or spindle positioning checkpoints as our data suggest both are functional in *kap123Δ* cells. Rather, genetic and physical interactions of Kap123p suggest this kap influences microtubule stability. It is unclear which microtubule regulatory pathway is affected or whether this defect is related to deficiencies in Kap123p-mediated nucleocytoplasmic transport. There is the potential that these findings reflect a direct role for a yeast kap in a biological process beyond nuclear transport. This would be consistent with non-transport roles identified for kaps in other organisms. Overall, these data have the potential to broaden our understanding both of kap function and microtubule regulation.

4.6 Future Directions

The identification of a functional role for Kap123p in microtubule regulation is intriguing. A number of experiments are required before the mechanism of this function can be elucidated. For example, further experimentation is required to test the physical interactions predicted by mass spectrometric analysis of eluted fractions from the Kap123-pA affinity precipitation experiment (*Table 3-2*; *Figure 3-9*). This can be achieved by Western blotting Kap123p-interacting fractions with antibodies directed against specific proteins. As discussed, there are a number of potential causes for the decreased microtubule stability observed in *kap123Δ* cells. Testable hypotheses can be proposed for each of the potential functions of Kap123p in microtubule organization discussed above. For example, the *kap123Δ* mutant is phenotypically similar to *gim* mutants. To determine whether Kap123p has a similar function leading to the formation of functional actin and tubulin, it may be advantageous to determine whether defects in actin organization exist in *kap123Δ* cells by evaluating whether these cells display sensitivity to the actin-binding drug latrunculin-A and by visualizing actin networks by fluorescence microscopy. Additionally, we obtained a wealth of data by investigating genetic interactions of *KAP123*. Using an unbiased approach such as SGA analysis may be a means of obtaining more information about Kap123p function. By using *KAP123* as a query gene, SGA provides a high-throughput, computerized approach for testing the viability and growth of double mutants lacking both *KAP123* and each of the non-essential yeast genes (Tong et al., 2001; Tong et al., 2004). By this method, it may be possible to fully elaborate the pathway through which Kap123p acts on microtubules.

CHAPTER FIVE: REFERENCES

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